# DRUG-OLIGOMER CONJUGATES

**Related Applications**

This application is a continuation-in-part application of U.S. Patent Application No. 09/474,915 (filed December 31, 1999, issued on March 30, 2004 as U.S. Patent No. 6,713,454; which claims priority from U.S. Provisional Application No. 60/153,649, filed September 13, 1999) and U.S. Patent Application No. 10/395,548 (filed March 24, 2003, currently pending; which is a continuation of U.S. Patent Application No. 09/802,739, filed March 9, 2001, now U.S. Patent No. 6,541,508, issued April 1, 2003; which is a continuation-in-part of U.S. Patent Application No. 09/476,974, filed December 31, 1999, now U.S. Patent No. 6,380,405, issued April 20, 2002), the disclosures of all of which are incorporated herein by reference in their entireties.

## 1. Background of the Invention

### 1.1 Field of the Invention

The present invention relates generally to drug-oligomer conjugates, pharmaceutical compositions comprising these drug-oligomer conjugates, and methods of making and using the conjugates and pharmaceutical compositions.

### 1.2 Related Art

The following is a discussion of art related to the present invention.

#### 1.2.1 Cancer of the Central Nervous System (CNS)

The American Cancer Society estimates that 16,800 people will be diagnosed with primary tumors of the central nervous system (CNS) in the United States in 1999, and that 13,100 of these will eventually die of their disease (American Cancer Society, 1999). Primary CNS tumors are among the most intractable cancers to treat. While such tumors rarely metastasize, their anatomic location causes a high incidence of morbidity and mortality resulting from compression of surrounding tissue as well as destruction of tissue invaded by such tumors. Additionally, CNS tumor cells often migrate away from the tumor to other locations in the brain. These migratory cells eventually form recurrent tumors. Multiple CNS tumors can also result from metastases of non-CNS neoplasmas.

Standard treatment for CNS tumors includes surgery and radiation therapy. However, with regard to surgery, complete surgical resection is often not possible. Normal brain tissue adjacent to the tumor is often critical to the survival or the quality of life of the patient. Consequently, survival of patients treated by surgery is low. The problems associated with radiation therapy are widely known in the art.

Systemic chemotherapy, would be a valuable therapeutic option for CNS tumors due to its ability to target microscopic deposits of tumor cells, including cells missed by radiation and surgery.

However, the inability to get such agents across the BBB has proven to be a significant limitation to the use of chemotherapy in the CNS. Many agents with activity against tumors do not cross the BBB to enter the brain parenchyma. Further, although many proven antineoplastic agents, including etoposide, will accumulate in brain tumors (Kiya, Uozumi et al. 1992), the concentration of these drugs decreases rapidly with distance from the tumor (Donelli, Zucchetti et al. 1992). In order to achieve effective treatment of the whole brain and expose microdeposits of tumor cells to drug, it would be desirable to have means for delivering therapeutic concentrations of anti-cancer drugs to the entire volume of the brain.

### 1.2.2 Barriers to CNS Drug Delivery

The brain is equipped with a barrier system which must be traversed to permit therapeutic drug delivery to the CNS. This brain barrier system has two major components: the choroid plexus and the blood-brain barrier (BBB). The choroid plexus separates cerebrospinal fluid (CSF) from the bloodstream, and the BBB separates brain interstitial fluid (ISF) from blood.

The BBB has about 1000 times more surface area than the choroid plexus and is the primary obstacle to delivery of therapeutic compounds to the CNS. The BBB acts as a selective partition, regulating the exchange of substances, between the CNS and the peripheral circulation. The primary structure of the BBB is the brain capillary endothelial wall. The tight junctions of brain capillary endothelial cells prevent circulating compounds from reaching the brain ISF by the paracellular route. Furthermore, recent work suggests the existence of a separate physiological barrier at the level of the basal lamina (Kroll et al. 1998). Other unique characteristics of the BBB include lack of intracellular fenestrations and pinocytic vesicles and a net negative charge on the luminal surface of the endothelium (Kroll et al. 1998).

The mechanisms by which substances traverse the BBB may generally be divided into active and passive transport mechanisms. Lipophilic molecules readily traverse the BBB by passive transport or diffusion through the endothelial plasma membranes, while hydrophilic molecules typically require an active transport system. Diffusion of many therapeutic compounds, across the BBB is also inhibited by size.

Many currently existing drug substances are unable to overcome these structural and metabolic barriers to enter the BBB in sufficient quantities to be efficacious. There is therefore a need for pharmaceutical compounds which can enable penetration of anti-cancer drugs through the BBB in sufficient amounts and at sufficient rates to be efficacious. Furthermore, many substances which, in theory, should be able to cross the BBB due to their lipophilicity, are not suitable for parenteral or oral delivery in the absence of potentially allergenic formulation ingredients. There is a need in the art for prodrugs which increase the solubility of drugs, preferably resulting in amphiphilic drugs which are orally available, soluble in the bloodstream, and which improve the ability of such drugs to enter the CNS. Conversely, there is a need in the art for prodrugs which increase the lipophilicity of hydrophilic drugs to produce amphiphilic prodrugs. Moreover, it is desirable that such amphiphilic prodrugs are hydrolyzed in vivo to release the active parent compound.

**1.2.3 Strategies for Delivering Therapeutic Compounds to the CNS**

Many attempts have been made in the art to deliver therapeutic compounds to the CNS with varying levels of success. Such attempts can generally be grouped into two categories: invasive and pharmacological.

Invasive delivery strategies include, for example, mechanical procedures, such as implantation of an intraventricular catheter, followed by pharmaceutical infusion into the ventricular compartment. Aside from general considerations relating to the invasiveness of mechanical procedures, a major difficulty with mechanical approaches is the lack of drug distribution. For example, injection of drugs into the CSF compartment commonly results in very little distribution beyond the surface of the brain. This lack of distribution is due in part to rapid exportation of drugs to the peripheral circulation.

Another invasive strategy for delivering therapeutic compounds to the CNS is by intracartoid infusion of highly concentrated osmotically active substances, such as mannitol or arabinose.

Their high local concentration causes shrinkages of the brain capillary endothelial cells, resulting in a transient opening of the tight junctions which enable molecules to traverse the BBB. Such procedures have considerable toxic effects, including inflammation, encephalitis, etc. Furthermore, such procedures are not selective: the opening of the tight junctions of the BBB permits many undesirable substances to cross the BBB along with the therapeutically beneficial molecule. For a recent review of osmotic opening and other invasive means for traversing the BBB, see Kroll, Neurosurgery, Vol. 42, No. 5, May 1998.

There is therefore a need in the art for means for selectively enabling therapeutic agents, such as peptides, to cross the BBB in a controlled manner which permits accumulation of sufficient quantities of the therapeutic in the brain to induce the desired therapeutic effect.

The present inventors have surprisingly discovered that conjugation of small amphiphilic polymers to drugs, such as etoposide, solves many of the aforementioned difficulties. This approach relies on rational oligomer design using a hydrophobic component plus a hydrophilic component to balance the physiochemical properties of the parent molecule. By varying the molecular weight of the hydrophobic and hydrophilic components of the oligomer and/or the molecular weight of the amphiphilic portion of the oligomer, the overall physiochemical profile of the conjugated molecule can be systematically adjusted to produce the desired degree of amphiphilicity with concomitant alterations in solubility and pharmacokinetics.

**1.2.4 Treatment for CNS Tumors**

The present inventors have surprisingly discovered that covalent modification of a hydrophobic drug with the oligomers of the present invention counteracts the hydrophobic nature of the parent compound and vastly improves its ability to penetrate the blood-brain barrier. Furthermore, the inventors have discovered that conjugation of a lipophilic parent drug, e.g., etoposide, with the oligomers of the present invention using a labile chemical bond that hydrolyzes in vivo, freeing the fully bioactive parent drug, improves solubility of the drug in the bloodstream and permits delivery of the drug to the CNS.

The prodrugs of the present invention exhibit the following useful properties:

The inactive prodrug form helps to mitigate administration-related toxicity;

A therapeutically significant amount of free drug reaches the CNS or other target;

It is expected that the prodrug can be delivered to the CNS via the oral route;

The prodrug is more readily formulated in hydrophilic formulations;

The half-life of elimination of the drug is extended; and

The conjugate has improved ability to pass from the intestine into the bloodstream.

**1.2.5 Etoposide as a Treatment for CNS Tumors**

Etoposide is a member of the epipodophyllotoxin class of compounds and is active against a broad spectrum of tumor types in vitro, including gliomas and astrocytomas (Giaccone, Gazdar et al. 1992; Kasahara, Fujiwara et al. 1992; Brown, McPherson et al. 1995; Chresta, Masters et al. 1996; Beauchesne, Bertrand et al. 1998). Etoposide has a molecular weight of 589 daltons, is lipophilic and is nearly insoluble in water (Hande 1998). As a result, it is typically formulated in a mixture of benzyl alcohol, polysorbate 80/Tween 80, polyethylene glycol 300 and ethanol (VePesid®, Bristol Myers Squibb). For administration VePesid is diluted in physiologically compatible solutions to 0.2 mg/mL to 0.4 mg/mL. Dose levels of 100 $mg/m^2$ to 600 $mg/m^2$ infused intravenously require volumes of 0.4 L to 5.1 L.

Although etoposide is hydrophobic, it does not readily penetrate the blood-brain barrier. As a result, concentrations of etoposide in brain parenchyma remain low after systemic administration (Hande, Wedlund et al. 1984; Donelli, Zucchetti et al. 1992; Kiya, Uozumi et al. 1992). Additionally, while etoposide can reach brain tumors, the concentration of etoposide in the tumor and surrounding tissue remain subtherapeutic (Donelli, Zucchetti et al. 1992). Due to the invasive nature of gliomas and astrocytomas and the difficulty of surgical resection, there is an urgent need for means for delivering drugs, such as etoposide, to areas of the brain that may harbor residual tumor cells.

One way to overcome the hydrophobicity of etoposide is to chemically modify the parent compound to increase its hydrophilicity. However, often such modifications destroy the desired biologic activity. Accordingly, there is a need in the art for amphiphilic etoposide prodrugs which increase penetration of the prodrug across the BBB and to thereby provide active etoposide to the CNS. Additionally, there is a need for a prodrug which facilitates

delivery of etoposide into the CNS by other routes of administration, including oral administration. In rodent biodistribution studies, I.V. etoposide is found at significant concentrations in most non-CNS tissues, especially liver and kidney. However, its accumulation in the CNS is very low (Hande, Wedlund et al. 1984; Donelli, Zucchetti et al. 1992). The hydrophobicity of etoposide makes it a good substrate for the P-glycoprotein multidrug pump or related drug transporters found in the endothelium of CNS blood vessels (Schinkel, Smit et al. 1994; Schinkel, Wagenaar et al. 1996). Experimental evidence suggests that these drug transporters act as the element of the blood-brain barrier responsible for actively barring etoposide from the CNS (Abe, Hasegawa et al. 1994). These drug transporters appear also to be partly responsible for clearance of etoposide and other drugs from the blood via direct extrusion through the intestinal wall into the lumen (Leu and Huang 1995; Mayer, Wagenaar et al. 1997; Sparreboom, van Asperen et al. 1997). There is an urgent need in the art for etoposide prodrugs which have been modified to permit the delivery of therapeutic concentrations of etoposide to the CNS by disabling export of the prodrugs by the P-glycoprotein multidrug pump and related drug transporting of the CNS epithelium.

A considerable amount of effort has been devoted to developing extended dosing regimens for etoposide (Greco, Johnson et al. 1991; Hande 1998). These efforts were based on clinical studies demonstrating the superior response rates obtained with 3 to 5 day schedules of administration versus 1 day (Cavalli, Sonntag et al. 1978). Accordingly, there is a need in the art for prodrug versions of the etoposide which extend the metabolic half-life of etoposide.

Etoposide has been shown to be effective in vitro at concentrations as low as 0.1 g/mL (Kasahara, Fujiwara et al. 1992). However, removal of etoposide reverses its inhibition of topoisomerase II, allowing cells to recover (Joel 1996). In addition, etoposide's effect on topoisomerase II is toxic primarily in the G2 phase of the cell cycle. As a result, a relatively brief treatment with etoposide will kill cells in G2 but allow cells in other phases of the cell cycle to recover. Accordingly, there is a need in the art for etoposide prodrugs which permit chronic dosing, thereby extending treatment of a cycling cell population and leading to cell death of a much greater proportion of the population.

Due to the relatively brief half-life of etoposide, daily (or more often) I.V. infusions are needed to maintain plasma etoposide concentrations in the therapeutic range. Thus, there is a need for etoposide prodrugs which permit an oral dosing regimen with its convenience and lower cost. As noted above, studies with oral etoposide have determined that its

bioavailability is erratic, leaving a physician with little assurance that the adequate dose had been delivered. Accordingly, a more consistently bioavailable etoposide prodrug is needed.

In summary, there is a need in the art for a means for producing consistent bioavailability of drugs, such as etoposide, without the necessity of multiple, prolonged I.V. infusions. Furthermore, there is a need for prodrugs which provide anti-cancer agents, such as etoposide, with a sufficiently extended plasma half-life to maintain in vivo concentration in a therapeutic range. Finally, there is a need in the art for prodrugs which enable effective oral delivery of drugs, such as etoposide, and which enables such orally delivered prodrugs to cross the BBB to enter the CNS.

**1.2.6 Toxicity and Formulation Problems Associated with Etoposide.**

Researchers have modified the physiochemical and pharmacologic properties etoposide by covalently attaching chemical moieties. Etoposide phosphate (Etopophos®, Bristol Myers Squibb) contains a phosphate group at the 4'-position of etoposide, resulting in a prodrug with increased aqueous solubility. The phosphate group hydrolyzes rapidly in vivo, and the compound has the same pharmacologic profile as etoposide. The improved water solubility of this etoposide analog increases the convenience of intravenous infusion; however, the oral bioavailability is only slightly improved, and the variability in bioavailability is still high, presumably because etoposide phosphate is converted into etoposide rapidly in the gut.

Several researchers have pursued formulation of cytotoxic drugs such as paclitaxel, adriamycin and doxorubicin in liposomal or micellar form. One objective of these strategies has been to create "sustained release" etoposide, producing extended periods of plasma etoposide concentrations in the therapeutic range. A second objective has been to sequester drugs in these vehicles to restrict access to non-target tissues. However, due to the large size of these particles, it is unlikely that this approach will facilitate improved oral bioavailability or penetration of the BBB. Accordingly, a need remains for etoposide prodrugs which facilitate improved oral bioavailability as well as penetration of the BBB.

A third approach is to conjugate the drug molecule to hydrophilic polymers to improve its solubility. Both polyethylene glycol (PEG) and polyglutamate have been used as hydrophilic polymers in this strategy. It appears that both polymers will increase the solubility of etoposide and therefore improve its handling characteristics. However, the conjugated

polymers have been relatively large, resulting in etoposide prodrugs with altered pharmacokinetics and low drug loading. There is therefore a need in the art for etoposide prodrugs with smaller polymers, which prodrugs have improved solubility characteristics as well as the other advantageous attributes described above.

## 2. Summary of the Invention

In one embodiment the invention provides a conjugate comprising at least one therapeutic compound and one or more PEG polymers and/or oligomers, each joined to a bonding site on the therapeutic compound by a hydrolyzable bond. The PEG polymers and/or oligomers each comprise a straight or branched PEG segment consisting of 1 to 25 polyethylene glycol units and a salt-forming moiety. In some embodiments the conjugate is a prodrug. In some embodiments the straight or branched PEG segment consists of from 2 to 20 polyethylene glycol units. In further embodiments the polyethylene glycol oligomer has a number of polyethylene glycol units selected from the group consisting of 1, 2, 3, 4, 5, 6, 7, 8, and 9. In some embodiments the salt-forming moiety is selected from the group consisting of: ammonium, carboxylate, phosphate, sulfate and mesylate. In some embodiments the therapeutic compound is derivatized by from 1 up to the maximum number of sites of attachment for the polyethylene glycol oligomer(s). When delivered via the oral route of administration to treat a mammalian subject having a disease condition responsive to the therapeutic compound, the invention can provide a therapeutically effective dose of the therapeutic compound to the blood. The therapeutic compound can be a peptide and/or protein.

In further embodiments the invention provides a pharmaceutical composition comprising a conjugate as described above and a pharmaceutically acceptable carrier. In some embodiments the conjugate is a prodrug. In some embodiments the pharmaceutical composition is in a form suitable for oral administration.

In further embodiments the invention provides a conjugate comprising a therapeutic compound joined by hydrolysable bond(s) to one or more PEG oligomer(s) selected from the group consisting of:

$$-\overset{\overset{\displaystyle O}{\|}}{C}-(CH_2)_n-\overset{\overset{\displaystyle R}{|}}{N}-CH_2CH_2(OC_2H_4)_mOCH_3 \qquad \text{(Formula 2)}$$

wherein n is from 1 to 7, m is from 2 to 25, and R is hydrogen or lower alkyl;

$$-\overset{\displaystyle O}{\overset{\|}{C}}-(CH_2)_n-\overset{\displaystyle O}{\overset{\|}{C}}-\overset{H}{N}-(CH_2)_p-\overset{\displaystyle R}{\overset{|}{N}}-CH_2CH_2(OC_2H_4)_mOCH_3 \qquad \text{(Formula 3)}$$

wherein n is from 1 to 6, p is from 2 to 8, m is from 2 to 25, and R is hydrogen or lower alkyl;

$$-\overset{\displaystyle O}{\overset{\|}{C}}-(CH_2)_n-\overset{\displaystyle O}{\overset{\|}{C}}-\overset{H}{N}-CH_2CH_2(OC_2H_4)_r-\overset{\displaystyle R}{\overset{|}{N}}-CH_2CH_2(OC_2H_4)_mOCH_3 \qquad \text{(Formula 4)}$$

wherein n is from 1 to 6, m and r are each independently from 2 to 25, and R is hydrogen or lower alkyl;

$$-\overset{\displaystyle O}{\overset{\|}{C}}-(CH_2)_n-\overset{\displaystyle O}{\overset{\|}{C}}-\overset{H}{N}-(CH_2)_p-\overset{\displaystyle R}{\overset{|}{N}}-CH_2CH_2(OC_2H_4)_mNH_2 \qquad \text{(Formula 5)}$$

wherein n is from 1 to 6, p is from 2 to 8, m is from 2 to 25 and R is hydrogen or lower alkyl;

$$-\overset{\displaystyle O}{\overset{\|}{C}}-(CH_2)_n-\overset{\displaystyle O}{\overset{\|}{C}}-\overset{H}{N}-(CH_2)_p-\overset{\displaystyle R}{\overset{|}{N}}-CH_2CH_2(OC_2H_4)_mNH_3^{+}X^{-} \qquad \text{(Formula 6)}$$

wherein n is from 1 to 6, p is from 2 to 8, m is from 2 to 25, R is hydrogen or lower alkyl, and $X^-$ is a negative ion;

$$-\overset{\displaystyle O}{\overset{\|}{C}}-(CH_2)_n-\overset{\displaystyle O}{\overset{\|}{C}}-\overset{H}{N}-(CH_2)_p-\overset{\displaystyle R^1}{\overset{|}{N}}-CH_2CH_2(OC_2H_4)_mNHR^2 \qquad \text{(Formula 7)}$$

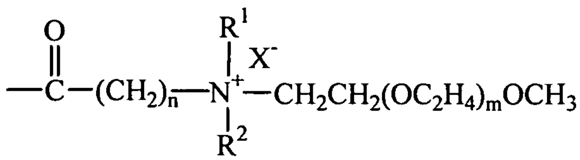
wherein n is from 1 to 6, p is from 2 to 8, m is from 2 to 25, and $R^1$ and $R^2$ are each independently hydrogen or lower alkyl;

$$-\overset{\displaystyle O}{\overset{\|}{C}}-(CH_2)_n-\overset{\displaystyle O}{\overset{\|}{C}}-\overset{H}{N}-CH_2CH_2(OC_2H_4)_mOCH_2-\overset{\displaystyle O}{\overset{\|}{C}}-NH(CH_2)_pN(CH_3)_2 \qquad \text{(Formula 8)}$$

wherein n is from 1 to 6, p is from 2 to 8 and m is from 2 to 25;

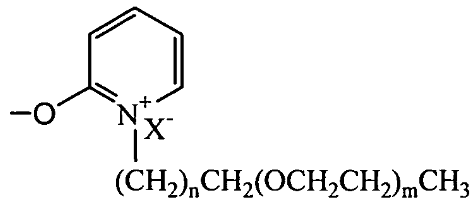
$$-\overset{\displaystyle O}{\overset{\|}{C}}-(CH_2)_n(OC_2H_4)_mO(CH_2)_p-\overset{\displaystyle O}{\overset{\|}{C}}-O^{-}X^{+} \qquad \text{(Formula 9)}$$

wherein n and p are each independently from 1 to 6, m is from 2 to 25 and $X^+$ is a positive ion;



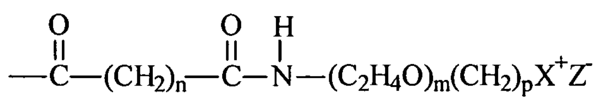
(Formula 10)

wherein n is from 1 to 5, m is from 2 to 25, $X^-$ is a negative ion, and wherein $R^1$ and $R^2$ are each independently hydrogen or lower alkyl;



(Formula 11)

wherein n is from 1 to 6, m is from 2 to 25 and $X^-$ is a negative ion; and



(Formula 12)

wherein n is from 1 to 12, m is from 2 to 25, p is from 2 to 12, $X^+$ is a positive ion and $Z^-$ is a negative ion.

In these embodiments the conjugate can be a prodrug. In further embodiments the therapeutic compound is derivatized by from 1 up to the maximum number of sites of attachment for the polyethylene glycol oligomer(s). In some embodiments the therapeutic compound is a peptide and/or protein.

The invention also provides a pharmaceutical composition comprising a conjugate, which comprises a therapeutic compound joined by hydrolysable bond(s) to one or more PEG oligomer(s) selected from the group consisting of formulae 2-12, and a pharmaceutically acceptable carrier. In some embodiments the conjugate is a prodrug. In some embodiments the pharmaceutical composition is in a form suitable for oral administration.

The invention also provides a method of treating a mammalian subject having a disease condition responsive to a therapeutic compound, said method comprising administering to the subject of an effective disease treating amount of a conjugate comprising at least one therapeutic compound and one or more PEG polymers and/or oligomers, each joined to a

bonding site on the therapeutic compound by a hydrolyzable bond. The PEG polymers and/or oligomers each comprise a straight or branched PEG segment consisting of 1 to 25 polyethylene glycol units and a salt-forming moiety. The conjugate can be a prodrug. The therapeutic compound can be a peptide and/or protein.

The invention also provides a method of treating a mammalian subject having a disease condition responsive to a therapeutic compound, said method comprising administering to the subject of an effective disease treating amount of a conjugate comprising the therapeutic compound joined by hydrolyzable bond(s) to one or more PEG oligomer(s) selected from the group consisting of:

$$-\overset{\displaystyle O}{\overset{\|}{C}}-(CH_2)_n-\overset{\displaystyle R}{\overset{|}{N}}-CH_2CH_2(OC_2H_4)_mOCH_3 \qquad \text{(Formula 2)}$$

wherein n is from 1 to 7, m is from 2 to 25, and R is hydrogen or lower alkyl;

$$-\overset{\displaystyle O}{\overset{\|}{C}}-(CH_2)_n-\overset{\displaystyle O}{\overset{\|}{C}}-\overset{\displaystyle H}{N}-(CH_2)_p-\overset{\displaystyle R}{\overset{|}{N}}-CH_2CH_2(OC_2H_4)_mOCH_3 \qquad \text{(Formula 3)}$$

wherein n is from 1 to 6, p is from 2 to 8, m is from 2 to 25, and R is hydrogen or lower alkyl;

$$-\overset{\displaystyle O}{\overset{\|}{C}}-(CH_2)_n-\overset{\displaystyle O}{\overset{\|}{C}}-\overset{\displaystyle H}{N}-CH_2CH_2(OC_2H_4)_r-\overset{\displaystyle R}{\overset{|}{N}}-CH_2CH_2(OC_2H_4)_mOCH_3 \qquad \text{(Formula 4)}$$

wherein n is from 1 to 6, m and r are each independently from 2 to 25, and R is hydrogen or lower alkyl;

$$-\overset{\displaystyle O}{\overset{\|}{C}}-(CH_2)_n-\overset{\displaystyle O}{\overset{\|}{C}}-\overset{\displaystyle H}{N}-(CH_2)_p-\overset{\displaystyle R}{\overset{|}{N}}-CH_2CH_2(OC_2H_4)_mNH_2 \qquad \text{(Formula 5)}$$

wherein n is from 1 to 6, p is from 2 to 8, m is from 2 to 25 and R is hydrogen or lower alkyl;

$$-\overset{\displaystyle O}{\overset{\|}{C}}-(CH_2)_n-\overset{\displaystyle O}{\overset{\|}{C}}-\overset{\displaystyle H}{N}-(CH_2)_p-\overset{\displaystyle R}{\overset{|}{N}}-CH_2CH_2(OC_2H_4)_mNH_3^+X^- \qquad \text{(Formula 6)}$$

wherein n is from 1 to 6, p is from 2 to 8, m is from 2 to 25, R is hydrogen or lower alkyl, and $X^-$ is a negative ion;

$$-\overset{\displaystyle O}{\overset{\|}{C}}-(CH_2)_n-\overset{\displaystyle O}{\overset{\|}{C}}-\overset{\displaystyle H}{N}-(CH_2)_p-\overset{\displaystyle R^1}{\overset{|}{N}}-CH_2CH_2(OC_2H_4)_mNHR^2 \qquad \text{(Formula 7)}$$

wherein n is from 1 to 6, p is from 2 to 8, m is from 2 to 25, and $R^1$ and $R^2$ are each independently hydrogen or lower alkyl;

$$-\overset{O}{\overset{\|}{C}}-(CH_2)_n-\overset{O}{\overset{\|}{C}}-\overset{H}{N}-CH_2CH_2(OC_2H_4)_mOCH_2-\overset{O}{\overset{\|}{C}}-NH(CH_2)_pN(CH_3)_2 \qquad \text{(Formula 8)}$$

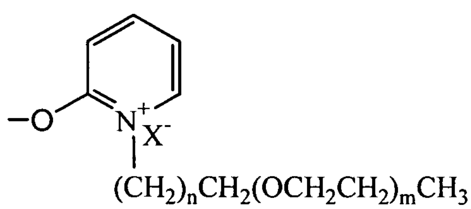
wherein n is from 1 to 6, p is from 2 to 8 and m is from 2 to 25;

$$-\overset{O}{\overset{\|}{C}}-(CH_2)_n(OC_2H_4)_mO(CH_2)_p-\overset{O}{\overset{\|}{C}}-O^-X^+ \qquad \text{(Formula 9)}$$

wherein n and p are each independently from 1 to 6, m is from 2 to 25 and $X^+$ is a positive ion;

$$-\overset{O}{\overset{\|}{C}}-(CH_2)_n-\overset{R^1}{\underset{R^2}{N^+}}\overset{X^-}{}-CH_2CH_2(OC_2H_4)_mOCH_3 \qquad \text{(Formula 10)}$$

wherein n is from 1 to 5, m is from 2 to 25, $X^-$ is a negative ion, and wherein $R^1$ and $R^2$ are each independently hydrogen or lower alkyl;



(Formula 11)

wherein n is from 1 to 6, m is from 2 to 25 and $X^-$ is a negative ion; and

$$-\overset{O}{\overset{\|}{C}}-(CH_2)_n-\overset{O}{\overset{\|}{C}}-\overset{H}{\overset{|}{N}}-(C_2H_4O)_m(CH_2)_pX^+Z^- \qquad \text{(Formula 12)}$$

wherein n is from 1 to 12, m is from 2 to 25, p is from 2 to 12, $X^+$ is a positive ion and $Z^-$ is a negative ion. The conjugate can be a prodrug. The therapeutic compound can be a peptide and/or protein.

**2.1 Definitions**

The terminology used in the description of the invention is for the purpose of describing particular embodiments only and is not intended to be limiting of the invention. As used herein, the following terms have the meanings indicated:

The singular forms "a," "an," and "the" are intended to include the plural forms as well, unless the context clearly indicates otherwise.

As used herein, the term "lower alkyl" refers to a straight or branched chain hydrocarbon having from one to 8 carbon atoms.

As used herein the terms "PEG" refers to straight or branched polyethylene glycol polymers and monomers. The term "PEG-oligomer" refers to oligomers comprising PEG polymers which have been modified to include groups which do not eliminate the amphiphilic properties of such polymers, e.g., without limitation, alkyl, aryl, amino-alkyl and amino-aryl; see also Formulae 2–12 set forth in Section 4, "Detailed Description of the Invention." The term "PEG subunit" or "PEG unit" refers to a single polyethylene glycol unit, i.e.: -($CH_2CH_2O$)-. The term "PEG oligomer/polymer" as used herein refers to PEG oligomers and/or PEG polymers.

As used herein terms such as "non-hydrolyzable" and phrases such as "not hydrolyzable" are used to refer to bonds which cannot be hydrolyzed under normal physiological conditions, as well as bonds which are not quickly hydrolyzed under normal physiological conditions, such as carbamate and amide bonds. The term "hydrolyzable" refers to bonds which are hydrolyzed under normal physiological conditions, such as ester and carbonate bonds.

A "therapeutically effective" amount or dose is an amount or dose which prevents, delays or reduces the severity of the onset of disease or other adverse medical condition, or an amount necessary to arrest or reduce the severity of an ongoing disease or other adverse medical condition, and also includes an amount necessary to enhance normal physiological functioning. The therapeutically effective amount or dose will vary with the age, general condition of the subject, the severity of the condition being treated, the particular biologically active agent administered, the duration of the treatment, the nature of any concurrent treatment, the pharmaceutically acceptable carrier used, and like factors within the

knowledge and expertise of those skilled in the art. As appropriate, a therapeutically effective amount or dose in any individual case can be determined by one of ordinary skill in the art by reference to the pertinent texts and literature and/or by using routine experimentation. (*See*, for example, Remington, *The Science And Practice of Pharmacy* (20th ed. 2000).

As used herein, "pharmaceutically acceptable" component (such as a salt, carrier, excipient or diluent) of a formulation according to the present invention is a component which (1) is compatible with the other ingredients of the formulation in that it can be combined with the prodrugs of the present invention without eliminating the biological activity of the prodrugs; and (2) is suitable for use in animals (including humans) without undue adverse side effects (such as toxicity, irritation, and allergic response). Side effects are "undue" when their risk outweighs the benefit provided by the pharmaceutical composition. Examples of pharmaceutically acceptable components include, without limitation, any of the standard pharmaceutical carriers such as phosphate buffered saline solutions, water, emulsions such as oil/water emulsion, microemulsions and various types of wetting agents.

Prodrugs are defined herein as inactive or partially inactive chemical derivatives of drugs that are metabolized to yield the pharmacologically active drug.

**3. Brief Description of the Drawings**

Figure 1. Structure of etoposide showing reactive hydroxyl groups that can be used as sites of attachment for the oligomers of the present invention.

Figure 2. General scheme of the operation of the present invention. The PEG-oligomer/polymer is attached to the hydroxyl groups on etoposide via a labile ester bond. In the bloodstream, the hydrolyzable bond is cleaved, thereby freeing the active parent compound.

Figure 3. Cleavage of conjugates in an aqueous environment. Samples of purified etoposide conjugate in phosphate buffers show increasing rate of hydrolysis as the pH increases. At pH 5.9 approximately 10% of the conjugate is hydrolyzed in 28 hours, while at pH 7.4 approximately 40% is hydrolyzed in the same time period. At pH 8.0 hydrolysis is essentially complete after 20 hours. No significant side products were observed during this conversion.

Figure 4. Graph showing that when the etoposide conjugate was incubated in fresh rat plasma at 37°C hydrolysis of the conjugated etoposide and appearance of free etoposide showed reciprocal behavior, indicating that hydrolysis of the PEG-oligomer/polymer etoposide conjugate is complete and releases free, active etoposide.

Figure 5. Graph showing in vivo profile of rats given etoposide conjugate versus rats given etoposide. Conjugates of etoposide were administered I.V. to Sprague Dawley rats at 9 µmol/kg. Measurement of free etoposide in plasma at various time points demonstrated that peak plasma etoposide concentration was increased by 60% and the half-life was extended by approximately 50% in rats given the etoposide conjugate (curve I) versus rats given etoposide (curve II).

Figure 6. When the etoposide concentration in brain tissue was measured in these rats the data revealed that injection with the etoposide conjugate led to a 3-fold increase in accumulation of free etoposide in the brain parenchyma, demonstrating an increased penetration of the blood-brain barrier by conjugated peptide hormones.

## 4. Detailed Description of the Invention

The ensuing detailed description is divided into sections for ease of reference only. Subject headings are not intended to limit the scope of the invention. Variations on the disclosed general synthetic methods will be readily apparent to those of ordinary skill in the art and are deemed to be within the scope of the present invention.

### 4.1 Prodrugs of the Invention

The present invention provides drug-PEG oligomer/polymer prodrugs. The prodrugs of the present invention generally comprise a drug component and a PEG-oligomer/polymer component. The prodrugs are useful in facilitating the formulation of drugs, the oral delivery of drugs, and the delivery of drugs across the BBB. These advantages are facilitated by increasing the amphiphilicity of the molecule. Thus, if the drug molecule is hydrophylic, the PEG-oligomer/polymer increases lipophilicity and thereby improves the amphiphilicity of the prodrug. Conversely, where the drug is lipophilic, the PEG-oligomer/polymer increases the hydrophilicity of the drug and thereby provides a more amphiphilic prodrug. The PEG-

oligomer/polymer is attached to the drug by a bond that hydrolyzes under normal physiological conditions to release the active parent compound.

### 4.1.1 Drug Component of the Prodrugs

The prodrugs of the present invention comprise a drug component. Suitable drug components are therapeutic compounds with free hydroxy, thio, phosphate or amino groups, which serve as sites of attachment for the oligomers. Preferred drug components are epipodophyllotoxins, and analogues thereof, such as etoposide, teniposide and etoposdide phosphate. The structural formula of etoposide is set forth in Figure 1.

In one aspect of the present invention, the drug component is an etoposide analogue. Many analogues of etoposide are known in the art, which are more or less effective than etoposide itself. The present invention contemplates the use of any etoposide analogue which does not have completely diminished activity.

Other drugs suitable for use in the prodrugs include paclitaxel and docetaxel. Still others are listed in US Patent 5,795,909, which is incorporated herein by reference.

### 4.1.2 PEG-Oligomers/Polymers

The amphiphilic PEG-oligomers/polymers of the prodrugs of the present invention may be straight or branched. Preferred oligomers/polymers comprise PEG polymers having from 2 to 25 PEG units, more preferably from 2 to 20 PEG units, still more preferably from 2 to 15 PEG units. Ideally, the PEG polymer has from 2 to 10 PEG units. In another aspect, the PEG polymer has a molecular weight which is not greater than 1000.

In a preferred mode, the PEG polymers have the formula:

$$-(CH_2CH_2O)_x-CH_3 \qquad \text{(Formula 1)}$$

wherein X = 2–25.

In a more preferred mode, X in Formula 1 is from 2 to 25, and in a still more preferred mode, from 2–20, still more preferably from 2–15, most preferably from 2–10. Ideally, X is 2, 3, 4, 5, 6, 7, 8, 9 or 10.

Preferred PEG oligomers are selected from the group consisting of:

$$-\overset{O}{\overset{\parallel}{C}}-(CH_2)_n-\overset{R}{\overset{|}{N}}-CH_2CH_2(OC_2H_4)_mOCH_3 \qquad \text{(Formula 2)}$$

wherein n is from 1 to 7, m is from 2 to 25, and R is hydrogen or a lower alkyl preferably selected from the group consisting of methyl, ethyl, propyl, isopropyl and t-butyl;

$$-\overset{O}{\overset{\parallel}{C}}-(CH_2)_n-\overset{O}{\overset{\parallel}{C}}-\overset{H}{N}-(CH_2)_p-\overset{R}{\overset{|}{N}}-CH_2CH_2(OC_2H_4)_mOCH_3 \qquad \text{(Formula 3)}$$

wherein n is from 1 to 6, p is from 2 to 8, m is from 2 to 25, and R is hydrogen or a lower alkyl, preferably selected from the group consisting of methyl, ethyl, propyl, isopropyl and t-butyl;

$$-\overset{O}{\overset{\parallel}{C}}-(CH_2)_n-\overset{O}{\overset{\parallel}{C}}-\overset{H}{N}-CH_2CH_2(OC_2H_4)_r-\overset{R}{\overset{|}{N}}-CH_2CH_2(OC_2H_4)_mOCH_3 \qquad \text{(Formula 4)}$$

wherein n is from 1 to 6, m and r are each independently from 2 to 25, and R is hydrogen or a lower alkyl, preferably selected from the group consisting of methyl, ethyl, propyl, isopropyl and t-butyl;

$$-\overset{O}{\overset{\parallel}{C}}-(CH_2)_n-\overset{O}{\overset{\parallel}{C}}-\overset{H}{N}-(CH_2)_p-\overset{R}{\overset{|}{N}}-CH_2CH_2(OC_2H_4)_mNH_2 \qquad \text{(Formula 5)}$$

wherein n is from 1 to 6, p is from 2 to 8 and m is from 2 to 25 and R is hydrogen or a lower alkyl, preferably selected from the group consisting of methyl, ethyl, propyl, isopropyl and t-butyl;

$$-\overset{O}{\overset{\parallel}{C}}-(CH_2)_n-\overset{O}{\overset{\parallel}{C}}-\overset{H}{N}-(CH_2)_p-\overset{R}{\overset{|}{N}}-CH_2CH_2(OC_2H_4)_mNH_3^+X^- \qquad \text{(Formula 6)}$$

wherein n is from 1 to 6, p is from 2 to 8, m is from 2 to 25, $X^-$ is a negative ion, preferably selected from the group consisting of chloro, bromo, iodo, phosphate, acetate, carbonate,

sulfate, tosylate and mesylate, and R is hydrogen or a lower alkyl, preferably selected from the group consisting of methyl, ethyl, propyl, isopropyl and t-butyl;

$$-\overset{O}{\overset{\|}{C}}-(CH_2)_n-\overset{O}{\overset{\|}{C}}-\overset{H}{N}-(CH_2)_p-\overset{R^1}{\overset{|}{N}}-CH_2CH_2(OC_2H_4)_mNHR^2 \qquad \text{(Formula 7)}$$

wherein n is from 1 to 6, p is from 2 to 8, m is from 2 to 25, and $R^1$ and $R^2$ are each independently a lower alkyl, preferably selected from the group consisting of methyl, ethyl, propyl, isopropyl, and t-butyl;

$$-\overset{O}{\overset{\|}{C}}-(CH_2)_n-\overset{O}{\overset{\|}{C}}-\overset{H}{N}-CH_2CH_2(OC_2H_4)_mOCH_2-\overset{O}{\overset{\|}{C}}-NH(CH_2)_pN(CH_3)_2 \qquad \text{(Formula 8)}$$

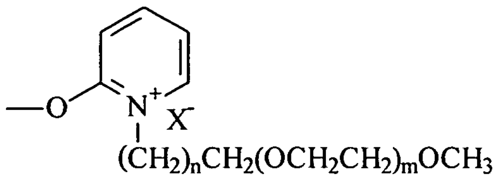
where n is from 1 to 6, p is from 2 to 8 and m is from 2 to 25;

$$-\overset{O}{\overset{\|}{C}}-(CH_2)_n(OC_2H_4)_mO(CH_2)_p-\overset{O}{\overset{\|}{C}}-O^-X^+ \qquad \text{(Formula 9)}$$

wherein n and p are each independently from 1 to 6, m is from 2 to 25 and $X^+$ is a positive ion, preferably selected from the group consisting of sodium, potassium, calcium, lithium and ammonium salts;

$$-\overset{O}{\overset{\|}{C}}-(CH_2)_n-\underset{R^2}{\overset{R^1\ X^-}{\overset{|}{N^+}}}-CH_2CH_2(OC_2H_4)_mOCH_3 \qquad \text{(Formula 10)}$$

wherein n is from 1 to 5, m is from 2 to 25, $X^-$ is a negative ion, preferably selected from the group consisting of: chloro, bromo, iodo, phosphate, acetate, carbonate, sulfate, tosylate, and mesylate, and wherein $R^1$ and $R^2$ are each independently hydrogen or lower alkyl and are preferably independently selected from the group consisting of hydrogen, methyl, ethyl propyl, isopropyl and t-butyl;

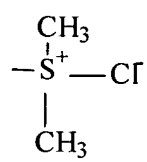


(Formula 11)

wherein n is from 1 to 6, m is from 2 to 25 and $X^-$ is a negative ion, preferably selected from the group consisting of: chloro, bromo, iodo, phosphate, acetate, carbonate, sulfate, tosylate, and mesylate; and

$$-\overset{\displaystyle O}{\overset{\|}{C}}-(CH_2)_n-\overset{\displaystyle O}{\overset{\|}{C}}-\overset{\displaystyle H}{\overset{|}{N}}-(C_2H_4O)_m(CH_2)_pX^+Z^- \qquad \text{(Formula 12)}$$

wherein n is from 1 to 12, m is from 1 to 25, p is from 2 to 12, $X^+$ is a positive ion and $Z^-$ is a negative ion. Preferably, n is from 2 to 8, and more preferably n is from 2 to 4. Preferably, p is from 2 to 8, and, more preferably, p is from 2 to 4. $X^+$ is preferably selected from the group consisting of $NH_3^+$ and trisubsitituted sulfur, namely sulfur substituted with one or more R' and R'' where R' and R'' each is methyl, ethyl, propyl and butyl. For example $X^+Z^-$ is



$Z^-$ is preferably selected from the group consisting of chloro anion, bromo anion, iodo anion, phosphate anion, acetate anion, trifluoracetate anion, carbonate anion, sulfate anion, and mesylate anion.

In any of the foregoing Formulae 1–12, the total number of PEG units is preferably from 2 to 25, more preferably from 2–20, still more preferably from 2–15, most preferably from 2–10. Ideally, the total number of PEG units is 2, 3, 4, 5, 6, 7, 8, 9 or 10. In formulae, such as Formula 4, which contain two PEG polymer segments, the preferred number of PEG units set forth in this paragraph may be contained completely in either of the two PEG polymer segments or may be distributed between the two PEG polymer segments.

The PEG-oligomer/polymer may also comprise one or more salt forming moieties. Preferred salt forming moieties are ammonium and carboxylate. Suitable salts also include any pharmaceutically acceptable acid-addition salts for PEG-oligomers/polymers having a basic amino group and pharmaceutically acceptable salts derived from pharmaceutically acceptable bases for PEG-oligomers/polymers having, e.g., a free carboxy group. Pharmaceutically acceptable salts of the acid may be prepared by treating the free acid with an appropriate base. Pharmaceutically acceptable base salts include, for example, alkali metal salts such as sodium or potassium, alkaline earth metal salts such as sodium or potassium, alkaline earth metal salts such as calcium or magnesium, and ammonium or alkyl ammonium salts.

### 4.1.3 Attachment of Lipophilic drug to PEG-Oligomers/Polymers

A key feature of the approach of the present invention is the nature of the chemical bond that joins the PEG-oligomer/polymer to etoposide. First, the parent drug must be protected and delivered by the oligomer, then hydrolysis of the joining bond must free the active parent drug. Second, the type of chemical bond used to join the two moieties should be chosen to enable in vivo hydrolysis, resulting in the release of a therapeutically effective amount of the active parent drug. Preferred hydrolyzable bonds include ester and carbonate.

The PEG-oligomers/polymers are suitably attached to the parent drug at any of the free hydroxy, thio, phosphate or amino groups of the parent drug compound. Figure 1 shows appropriate points of attachment for the oligomers to the etoposide molecule. It will be appreciated by those of skill in the art that a solution of prodrugs according to the present invention where the drug is etoposide or the like may comprise a mixture of 1-, 2-, and/or 3-substituted prodrugs.

### 4.2 Methods for Producing the Etoposide-PEG Conjugates

Etoposide is commercially available and can be prepared by known synthetic methods. In the ensuing examples, the n, p, m, R, and $R^1$ and $R^2$ symbols are as described above in general Formulae 1-12.

### 4.2.1 Formula 1

The polymers of Formula 1 are commercially available and/or are readily synthesized by one of skill in the art without undue experimentation.

**4.2.2 Formula 2**

In the synthesis of the oligomers of Formula 2:

$$-\overset{\overset{\displaystyle O}{\|}}{C}-(CH_2)_n-\overset{\overset{\displaystyle R}{|}}{N}-CH_2CH_2(OC_2H_4)_mOCH_3 \qquad \text{(Formula 2)}$$

wherein n is from 1 to 7, m is from 2 to 25, and R is hydrogen or a lower alkyl, it is desirable to start with an ester of a fatty acid having a terminal carbon which bears a primary amino moiety. Such compounds are commercially available. The amino ester in an inert solvent is treated with a solution of monomethoxy polyethylene glycol of appropriate molecular weight bearing an aldehyde terminal carbon, followed by the addition of a solution of sodium borohydride. The product is purified after solvent extraction by column chromatography.

$$CH_3CH_2O_2C(CH_2)_nNH_2 + O{=}CHCH_2(OC_2H_4)_mOCH_3$$

$$\Big\downarrow \begin{array}{l} \text{1. THF} \\ \text{2. } NaBH_4 \end{array}$$

$$CH_3CH_2O_2C(CH_2)_nNHCH_2CH_2(OC_2H_4)_mOCH_3$$

where n and m are as previously defined.

Sometimes it is desirable to alkylate the secondary amine moiety to form a desired oligomer bearing a tertiary amine. A solution of the oligomer in an inert solvent is treated with one equivalent of alkyl halide. The product is purified after solvent extraction by column chromatography.

$$CH_3CH_2O_2C(CH_2)_nNHCH_2CH_2(OC_2H_4)_mOCH_3$$

$$\Big\downarrow \text{RBr}$$

$$CH_3CH_2O_2C(CH_2)_nN(R)CH_2CH_2(OC_2H_4)_mOCH_3$$

The ester is converted to an acid by treating it in an inert solvent with a dilute solution of sodium hydroxide at room temperature. The free acid is purified after solvent extraction by column chromatography. The acid is coupled to the drug after in situ activation.

$$CH_3CH_2O_2(CH_2)_nN(R)CH_2CH_2(OC_2H_4)_mOCH_3$$

$$\Big\downarrow \text{NaOH}$$

$$HOC(O)(CH_2)_nN(R)CH_2CH_2(OC_2H_4)_mOCH_3$$

$$\Big\downarrow \begin{array}{l}\text{1. Drug–OH}\\ \text{2. dicyclohexylcarbinolamide (DCCE)}\\ \text{and 4-dimethylaminopyridine (DMAP)}\end{array}$$

$$\text{Drug–}OC(O)(CH_2)_nN(R)CH_2CH_2(OC_2H_4)_mOCH_3$$

The drug in these examples can be, for example, etoposide.

It is sometimes desirable to synthesize the drug-oligomer by starting with the therapeutic compound derivatized as an ester of fatty acid having a terminal carbon which bears a halide and an appropriate monomethoxy-polyethylene glycol with a terminal carbon bearing a primary amino moiety. The polyethylene glycol reagent is dissolved in an inert solvent at room temperature. An equivalent amount of the drug-halide is dissolved in an inert solvent and added slowly to the solution of polyethylene glycol. The product is purified after solvent extraction using column chromatography.

$$D\text{-}O_2C(CH_2)_nCH_2Br + NH_2CH_2CH_2(OC_2H_4)_mOCH_3 \xrightarrow{THF} D\text{-}O_2C(CH_2)_nCH_2NHCH_2CH_2(OC_2H_4)_mOCH_3$$

The ester is hydrolyzed with a dilute solution of sodium hydroxide as in the previous procedure and coupled to the drug (e.g., etoposide) after *in situ* activation as in the previous example.

**4.2.3 Formula 3**

In the synthesis of the oligomer of Formula 3:

$$-\overset{\overset{\large O}{\|}}{C}-(CH_2)_n-\overset{\overset{\large O}{\|}}{C}-\overset{\overset{}{H}}{N}-(CH_2)_p-\overset{\overset{R}{|}}{N}-CH_2CH_2(OC_2H_4)_mOCH_3 \qquad \text{(Formula 3)}$$

wherein n is from 1 to 6, p is from 2 to 8, m is from 2 to 25, and R is hydrogen or a lower alkyl, it is desirable to start with a half-ester of a dicarboxylic acid of an aliphatic compound and an amino-containing polyethylene. In the synthesis of the amino-containing polyethylene, an appropriate molecular weight monomethyl polyethylene glycol having an aldehyde moiety at the terminal end is treated in an inert solvent with an aliphatic compound bearing amino moieties at the two terminal carbons. One amino moiety is protected with tert-butoxycarbonyl while the free amine reacts with the aldehyde moiety. The product is purified after solvent extraction column chromatography. The product is deprotected by treating in an inert solvent with trifluoroacetic acid, neutralizing the acid and purifying after solvent extraction using column chromatography.

$$(CH_3)_3C\text{-}OC(O)NH(CH_2)_pNH_2 + O{=}CHCH_2(OC_2H_4)_mOCH_3$$

$$\downarrow$$

$$(CH_3)_3C\text{-}OC(O)NH(CH_2)_pN{=}CHCH_2(OC_2H_4)_mOCH_3$$

$$\downarrow \text{TFA}$$

$$NH_2(CH_2)_pN{=}CHCH_2(OC_2H_4)_mOCH_3$$

The half ester in an inert solvent is treated with a solution of the amino-derivatized polyethylene glycol at room temperature after an in situ activation of the acid. The product is purified by column chromatography after solvent extraction. The imino moiety is reduced by treating with a solution of sodium borohydride and purified as in the previous procedure.

It is sometimes desirable to alkylate the secondary amine. To achieve this end, the oligomer is dissolved in an inert solvent and treated with a solution of an alkyl halide in an inert solvent.

The ester is hydrolyzed, activated in situ, and coupled to the therapeutic compound (e.g., etoposide).

$$NH_2(CH_2)_pN{=}CHCH_2(OC_2H_4)_mOCH_3 + CH_3CH_2O_2C(CH_2)_nC(O)OH$$

$$\downarrow \text{DCC/DMAP}$$

$$CH_3CH_2O_2C(CH_2)_nC(O)NH(CH_2)_pN{=}CHCH_2(OC_2H_4)_mOCH_3$$

$$\downarrow NaBH_4/RBr$$

$$CH_3CH_2O_2C(CH_2)_nC(O)NH(CH_2)_pNRCH_2CH_2(OC_2H_4)_mOCH_3$$

$$\downarrow NaOH$$

$$HOC(O)(CH_2)_nC(O)NH(CH_2)_pNRCH_2CH_2(OC_2H_4)_mOCH_3$$

$$\big\downarrow \text{ D–OH/DCC/DMAP}$$

$$D\text{–}OC(O)(CH_2)_nC(O)NH(CH_2)_pNRCH_2CH_2(OC_2H_4)_mOCH_3$$

where D indicates the drug component of the drug-amphiphile conjugate. The amphiphilic drug conjugate is converted to a salt form to improve aqueous solubility as necessary using a pharmaceutically acceptable acid.

### 4.2.4 Formula 4

The procedure for the synthesis of the oligomer of Formula 4:

$$—\overset{\overset{\displaystyle O}{\|}}{C}—(CH_2)_n—\overset{\overset{\displaystyle O}{\|}}{C}—\overset{H}{N}—CH_2CH_2(OC_2H_4)_r—\overset{\overset{\displaystyle R}{|}}{N}—CH_2CH_2(OC_2H_4)_mOCH_3 \qquad \text{(Formula 4)}$$

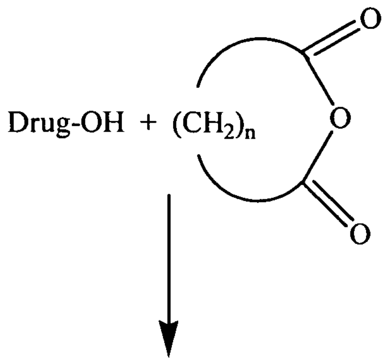
wherein n is from 1 to 6, m and r are each independently from 2 to 25, and R is hydrogen or a lower alkyl, is the same as for the oligomer of Formula 3 with the exception that the aliphatic diamino moieties are replaced with polyethylene glycol diamine.

### 4.2.5 Formula 5

In the synthesis of a prodrug comprising the oligomer of Formula 5:

$$—\overset{\overset{\displaystyle O}{\|}}{C}—(CH_2)_n—\overset{\overset{\displaystyle O}{\|}}{C}—\overset{H}{N}—(CH_2)_p—\overset{\overset{\displaystyle R}{|}}{N}—CH_2CH_2(OC_2H_4)_mNH_2 \qquad \text{(Formula 5)}$$

wherein n is from 1 to 6, p is from 2 to 8 and m is from 2 to 25 and R is hydrogen or a lower alkyl, the drug bearing a hydroxyl moiety is treated in an inert solvent with an aliphatic acid anhydride to form a half ester. The half-ester is dissolved in an inert solvent, activated and treated with one equivalent of a polyethylene glycol of appropriate molecular weight, in which the terminal hydroxyl moieties are replaced with amino moieties.



Drug-OC(O)$(CH_2)_n$C(O)OH

$\downarrow$ 1. 1,1'-carbonyldiimidazole (CDI)
2. $NH_2(CH_2)_pNRCH_2CH_2(OC_2H_4)_mNHC(O)OC(CH_3)_3$
3. TFA/Basic Column

Drug-OC(O)$(CH_2)_n$C(O)NH$(CH_2)_pNRCH_2CH_2(OC_2H_4)_mNH_2$

where all substituent groups (e.g., n, m and p) are as previously defined.

**4.2.6 Formula 6**

The oligomer of Formula 6:

$$-\overset{\overset{\displaystyle O}{\|}}{C}-(CH_2)_n-\overset{\overset{\displaystyle O}{\|}}{C}-\overset{H}{N}-(CH_2)_p-\overset{\overset{\displaystyle R}{|}}{N}-CH_2CH_2(OC_2H_4)_mNH_3^+X^- \qquad \text{(Formula 6)}$$

wherein n is from 1 to 6, p is from 2 to 8, m is from 2 to 25, R is hydrogen or a lower alkyl, $X^-$ is a negative ion, is prepared by treating the compound represented by Formula 5 with a pharmaceutically acceptable acid to obtain the appropriate salt. The salt increases the water-solubility of the amphiphilic drug conjugate.

**4.2.7 Formula 7**

The synthesis of the oligomer of Formula 7:

$$-\overset{\overset{\displaystyle O}{\|}}{C}-(CH_2)_n-\overset{\overset{\displaystyle O}{\|}}{C}-\overset{H}{N}-(CH_2)_p-\overset{\overset{\displaystyle R^1}{|}}{N}-CH_2CH_2(OC_2H_4)_mNHR^2 \qquad \text{(Formula 7)}$$

wherein n is from 1 to 6, p is from 2 to 8, m is from 2 to 25, and $R^1$ and $R^2$ are each independently a lower alkyl, is analogous to the synthesis of the oligomer of Formula 5, with the exception that the end-terminal amino moiety is alkylated with the halide of a short chain alkyl group such as methyl, ethyl, propyl, isopropyl or t-butyl before reacting to the half-ester of the drug.

$$(CH_3)_3COC(O)NH_2(CH_2)_pBr + NH_2CH_2CH_2(OC_2H_4)_mNH_2$$

$$\downarrow$$

$$(CH_3)_3COC(O)NH_2(CH_2)_pNHCH_2CH_2(OC_2H_4)_mNH_2$$

$$\downarrow \quad \begin{array}{ll} 1. & \mathrm{RBr} \\ 2. & \mathrm{TFA} \\ 3. & \text{Basic Column} \\ 4. & \mathrm{D{-}O_2C(CH_2)_nCOOH/DCC} \end{array}$$

$$D{-}OC(O)(CH_2)_nC(O)NH(CH_2)_pNRCH_2CH_2(OC_2H_4)_mNHR$$

where n, m and R are as previously defined.

**4.2.8 Formula 8**

In the synthesis of the oligomer of Formula 8:

$$-\overset{\overset{\displaystyle O}{\|}}{C}-(CH_2)_n-\overset{\overset{\displaystyle O}{\|}}{C}-\overset{\displaystyle H}{N}-CH_2CH_2(OC_2H_4)_mOCH_2-\overset{\overset{\displaystyle O}{\|}}{C}-NH(CH_2)_pN(CH_3)_2 \qquad \text{(Formula 8)}$$

wherein n is from 1 to 6, p is from 2 to 8 and m is from 2 to 25, the half-ester of the aliphatic dicarboxylic acid is treated in an inert solvent with polyethylene glycol that has already been derivatized with amino moieties, after in situ activation.

$$CH_3CH_2OC(O)(CH_2)_nC(O)OH$$

$$+$$

$$NH_2CH_2CH_2(OC_2H_4)_mOCH_2C(O)NH(CH_2)_pN(CH_3)_2$$

$$\Big\downarrow \text{ 1. DCC; 2. NaOH}$$

$$HOC(O)(CH_2)_nC(O)NHCH_2CH_2(OC_2H_4)_mOCH_2C(O)NH(CH_2)_pN(CH_3)_2$$

The amino-derivatized polyethylene glycol is prepared from an N-protected polyethylene glycol amino acid.

$$\text{tert-BOCNHCH}_2\text{CH}_2(\text{OC}_2\text{H}_4)_m\text{OCH}_2\text{C(O)–OH} + \text{NH}_2(\text{CH}_2)_p\text{N(CH}_3)_2$$

$$\Big\downarrow \text{ DCC/DMAP}$$

$$\text{tert-BOCNHCH}_2\text{CH}_2(\text{OC}_2\text{H}_4)_m\text{OCH}_2\text{C(O)–NH(CH}_2)_p\text{N(CH}_3)_2$$

$$\Big\downarrow \text{ TFA/Basic Column}$$

$$NH_2CH_2CH_2(OC_2H_4)_mOCH_2C(O){-}NH(CH_2)_pN(CH_3)_2$$

The primary amino moiety is deprotected with trifluoroacetic acid and basified before treating with the half-ester.

**4.2.9 Formula 9**

In the synthesis of the oligomer of Formula 9:

$$-\overset{\overset{\displaystyle O}{\|}}{C}-(CH_2)_n(OC_2H_4)_mO(CH_2)_p-\overset{\overset{\displaystyle O}{\|}}{C}-O^-X^+ \qquad \text{(Formula 9)}$$

wherein n and p are each independently from 1 to 6, m is from 2 to 25 and $X^+$ is a positive ion, the starting acid is commercially available. It is sometimes desirable to prepare the diacid. To achieve this end, the appropriate modified polyethylene glycol oligomer is treated in an inert solvent with sodium hydride and an ester of a fatty acid bearing a halide moiety at the terminal carbon. The carboxylic acid diester is hydrolyzed in a dilute solution of sodium

hydroxide and coupled to the drug moiety after in situ activation. The desired product is separated and purified by column chromatography

$$CH_3CH_2O_2(CH_2)_nBr + HOCH_2CH_2(OC_2H_4)_mO(CH_2)_pCO_2CH_2CH_3$$

$$\Big\downarrow \text{2NaH/THF}$$

$$CH_3CH_2O_2C(CH_2)_n(OC_2H_4)_mO(CH_2)_pCO_2CH_2CH_3$$

$$\Big\downarrow \text{2NaH/THF}$$

$$HOC(O)(CH_2)_n(OC_2H_4)_mO(CH_2)_pC(O)OH$$

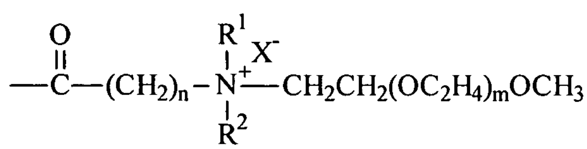
$$\Big\downarrow \begin{array}{l}\text{Drug–OH/}CH_2Cl_2\\ \text{DCC/DMAP}\end{array}$$

$$\text{Drug–}OC(O)(CH_2)_n(OC_2H_4)_mO(CH_2)_pC(O)OH$$

where n, m and p are as previously defined.

**4.2.10 Formula 10**

The synthesis of the oligomer of Formula 10:

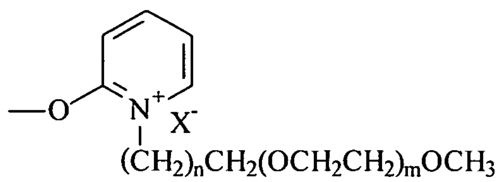


(Formula 10)

wherein n is from 1 to 5, m is from 2 to 25, $X^-$ is a negative ion, and wherein $R^1$ and $R^2$ are each independently hydrogen or lower alkyl, is analogous to the synthesis of the oligomer of Formula 2, with the exception that the amino moiety is quaternized with short-chain aliphatic moieties. It is noted that the methoxy moiety can include other short chain (1 to 6 carbons) aliphatic moieties.

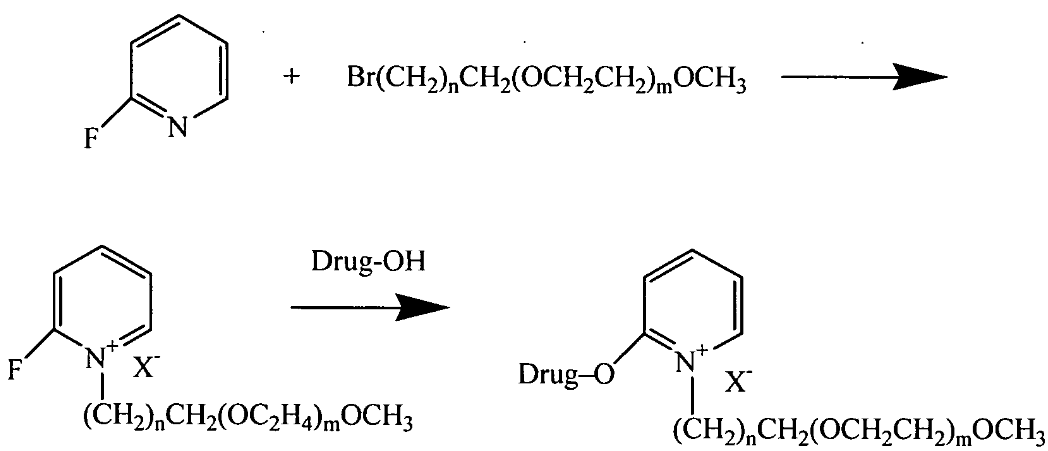
**4.2.11 Formula 11**

In the synthesis of the oligomers of Formula 11:



(Formula 11)

wherein n is from 1 to 6, m is from 2 to 25 and $X^-$ is a negative ion, a 2-fluoro- or 4-fluoro-pyridine is treated in an inert solvent with a monomethoxypolyethylene glycol having a terminal carbon bearing a halide, tosylate or mesylate ion. This pyridinium derivative is precipitated and triturated with an appropriate solvent and dried. The salt in an inert solvent is treated with drug, such as paclitaxel or docetaxel, in the presence of a quaternary-salt compound forming base, to yield a polyethylene glycol pyridinium derivative.



**4.2.12 Formula 12**

Procedures for preparing the oligomers of Formula 12 are described in the Examples of Section 6 below.

**4.2.13 Attachment of PEG Polymers/Oligomers to the Therapeutic Compound**

The following procedure is illustratively given using etoposide as an example; however, the procedure will be understood by those of skill in the art to have applicability to other

therapeutic compounds as well. The PEG-oligomers/polymers of the present invention can be attached to etoposide according to the following general synthetic procedure. Etoposide is dissolved in a substantially dry organic solvent, e.g., chloroform. Pyridine or another quaternary-compound forming agent is added to the foregoing mixture. Activated PEG-oligomer/polymer is added dropwise and the mixture is stirred for 3–5 hours. Then reaction mixture is washed with 1% $H_2SO_4$ and deionized water, dried over $MgS0_4$ and concentrated. The residue is chromatographed on silica gel column, using for example, chloroform-methanol (90%-10%) as developing agent. The fractions containing the desired prodrugs are collected, concentrated, and dried. Product is characterized by TLC, HPLC, NMR, and/or MS.

### 4.3 Pharmaceutical Compositions and Methods of Use

The pharmaceutical compositions containing the novel prodrugs as active ingredients may be any pharmaceutically acceptable dosage forms known in the art which do not completely diminish the activity of the prodrugs. Examples include oral, injectable or I.V. dosage forms. Each dosage form comprises an effective amount of a prodrug and pharmaceutically inert ingredients, e.g., conventional excipients, vehicles, fillers, binders, disintegrants, solvents, solubilizing agents, sweeteners, coloring agents and any other inactive ingredients which are regularly included in pharmaceutical dosage forms. Suitable oral dosage forms include tablets, capsules, caplets, gelcaps, pills, liquid solutions, suspensions or elixirs, powders, lozenges, micronized particles and osmotic delivery systems. Suitable injectable and IV dosage forms include isotonic saline solutions or dextrose solutions containing suitable buffers and preservatives. Many such dosage forms and vehicles, and listings of inactive ingredients therefor, are well-known in the art and are set forth in standard texts such as *The Pharmaceutical Codex: Principles and Practice of Pharmaceutics*, 12$^{th}$ edition (1994).

The present invention also comprehends a method of treating a mammalian subject having a tumor, cancer, or other disease condition responsive to the specific therapeutic compound (e.g., an anti-cancer drug such as etoposide). The method comprises administering to said subject a pharmaceutical composition containing a pharmaceutically effective amount of the drug-oligomer prodrug according to the present invention. Diseases/conditions which may be treated by the invention include, without limitation, cancers, tumors and malignancies. Diseases/conditions which may be treated by the invention also include, without limitation, small cell lung cancer, non-small cell lung cancer, testicular cancer, lymphoma, leukemia,

ovarian cancer (specifically including relapsed or platinum refractory ovarian cancer), and gastric cancer.

The prodrugs of the present invention can be administered in such oral (including buccal and sublingual) dosage forms as tablets, capsules (each including timed release and sustained release formulations), pills, powders, granules, elixirs, tinctures, suspensions, syrups and emulsions. Likewise, they may also be administered in nasal, ophthalmic, otic, rectal, topical, intravenous (both bolus and infusion), intraperitoneal, intraarticular, subcutaneous or intramuscular inhalation or insufflation form, all using forms well known to those of ordinary skill in the pharmaceutical arts.

The dosage regimen utilizing the prodrugs of the present invention is selected in accordance with a variety of factors including type, species, age, weight, sex and medical condition of the patient; the severity of the condition to be treated; the route of administration; the renal and hepatic function of the patient; and the particular compound or salt thereof employed. An ordinarily skilled physician or veterinarian can readily determine and prescribe the effective amount of the drug required to prevent, counter or arrest the progress of the condition.

Oral administration is generally preferred for administration to a human. In some cases, a relatively lower dose is sufficient and, in some cases, a relatively higher dose or increased nurnber of doses may be necessary. Topical application similarly may be once or more than once per day depending upon the usual medical considerations. Advantageously, prodrugs of the present invention may be administered in a single daily dose, or the total daily dosage may be administered in divided doses of two, three or four times daily.

In the methods of the present invention, the prodrugs can form the active ingredient and are typically administered in admixture with suitable pharmaceutical diluents, excipients or carriers (collectively referred to herein as "carrier" materials) suitably selected with respect to the intended form of administration, that is, oral tablets, capsules, elixirs, syrups and the like, and consistent with conventional pharmaceutical practices.

For instance, for oral administration in the form of a tablet or capsule, the active drug component can be combined with an oral, non-toxic pharmaceutically acceptable inert carrier such as ethanol, glycerol, water and the like. Powders are prepared by comminuting the compound to a suitable fine size and mixing with a similarly comminuted pharmaceutical

carrier such as an edible carbohydrate, as, for example, starch or mannitol. Flavoring, preservative, dispersing and coloring agent can also be present.

Capsules are made by preparing a powder mixture as described above and filling formed gelatin sheaths. Glidants and lubricants such as colloidal silica, talc, magnesium stearate, calcium stearate or solid polyethylene glycol can be added to the powder mixture before the filling operation. A disintegrating or solubilizing agent such as agar-agar, calcium carbonate or sodium carbonate can also be added to improve the availability of the medicament when the capsule is ingested.

Moreover, when desired or necessary, suitable binders, lubricants, disintegrating agents and coloring agents can also be incorporated into the mixture. Suitable binders include starch, gelatin, natural sugars such as glucose or beta-lactose, corn sweeteners, natural and synthetic gums such as acacia, tragacanth or sodium alginate, carboxymethylcellulose, polyethylene glycol, waxes and the like. Lubricants used in these dosage forms include sodium oleate, sodium stearate, magnesium stearate, sodium benzoate, sodium acetate, sodium chloride and the like. Disintegrators include, without limitation, starch, methyl cellulose, agar, bentonite, xanthan gum and the like. Tablets are formulated, for example, by preparing a powder mixture, granulating or slugging, adding a lubricant and disintegrant and pressing into tablets. A powder mixture is prepared by mixing the drug-oligomer prodrug, suitably comminuted, with a diluent or base as described above, and optionally, with a binder such as carboxymethylcellulose, an aliginate, gelatin, or polyvinyl pyrrolidone, a solution retardant such as paraffin, a resorption accelerator such as a quaternary salt and/or an absorption agent such as bentonite, kaolin or dicalcium phosphate. The powder mixture can be granulated by wetting with a binder such as syrup, starch paste, acadia mucilage or solutions of cellulosic or polymeric materials and forcing through a screen. As an alternative to granulating, the powder mixture can be run through the tablet machine and the result is imperfectly formed slugs broken into granules. The granules can be lubricated to prevent sticking to the tablet forming dies by means of the addition of stearic acid, a stearate salt, talc or mineral oil. The lubricated mixture is then compressed into tablets. The prodrugs of the present invention can also be combined with free flowing inert carrier and compressed into tablets directly without going through the granulating or slugging steps. A clear or opaque protective coating consisting of a sealing coat of shellac, a coating of sugar or polymeric material and a polish

coating of wax can be provided. Dyestuffs can be added to these coatings to distinguish different unit dosages.

Oral fluids such as solution, syrups and elixirs can be prepared in dosage unit form so that a given quantity contains a predetermined amount of the compound. Syrups can be prepared by dissolving the compound in a suitably flavored aqueous solution, while elixirs are prepared through the use of a non-toxic alcoholic vehicle. Suspensions can be formulated by dispersing the compound in a non-toxic vehicle. Solubilizers and emulsifiers such as ethoxylated isostearyl alcohols and polyoxy ethylene sorbitol ethers, preservatives, flavor additive such as peppermint oil or saccharin, and the like can also be added.

Where appropriate, dosage unit formulations for oral administration can be microencapsulated. The formulation can also be prepared to prolong or sustain the release as for example by coating or embedding particulate material in polymers, wax or the like.

The prodrugs of the present invention can also be administered in the form of liposome delivery systems, such as small unilamellar vesicles, large unilamellar vesicles and multilamellar vesicles. Liposomes can be formed from a variety of phospholipids, such as cholesterol, stearylamine or phosphatidylcholines.

Prodrugs of the present invention may also be delivered by the use of monoclonal antibodies as individual carriers to which the compound molecules are coupled. The prodrugs of the present invention may also be coupled with soluble polymers, such as targetable drug carriers. Such polymers can include polyvinylpyrrolidone, pyran copolymer, polyhydroxypropylmethacrylamide- phenol, polyhydroxyethylaspartamidephenol, or polyethyleneoxidepolylysine substituted with palmitoyl residues. Furthermore, the prodrugs of the present invention may be coupled to a class of biodegradable polymers useful in achieving controlled release of a drug, for example, polylactic acid, polepsilon caprolactone, polyhydroxy butyric acid, polyorthoesters, polyacetals, polydihydropyrans, polycyanoacrylates and cross-linked or amphipathic block copolymers of hydrogels.

The present invention includes pharmaceutical compositions containing about 0.01 to about 99.5%, more particularly, about 0.5 to about 90% of a drug-oligomer prodrug in combination with a pharmaceutically acceptable carrier.

Parenteral administration can be effected by utilizing liquid dosage unit forms such as sterile solutions and suspensions intended for subcutaneous, intramuscular or intravenous injection. These are prepared by suspending or dissolving a measured amount of the drug-oligomer prodrug in a non-toxic liquid vehicle suitable for injection such as aqueous oleaginous medium and sterilizing the suspension or solution.

Alternatively, a measured amount of the drug-oligomer prodrug is placed in a vial and the vial and its contents are sterilized and sealed. An accompanying vial or vehicle can be provided for mixing prior to administration. Non-toxic salts and salt solutions can be added to render the injection isotonic. Stabilizers, preservations and emulsifiers can also be added.

Rectal administration can be effected utilizing suppositories in which the drug-oligomer prodrug is admixed with low-melting water-soluble or insoluble solids such as polyethylene glycol, cocoa butter, higher ester as for example flavored aqueous solution, while elixirs are prepared through myristyl palmitate or mixtures thereof.

Topical formulations of the present invention may be presented as, for instance, ointments, creams or lotions, eye ointments and eye or ear drops, impregnated dressings and aerosols, and may contain appropriate conventional additives such as preservatives, solvents to assist drug penetration and emollients in ointments and creams. The formulations may also contain compatible conventional carriers, such as cream or ointment bases and ethanol or oleyl alcohol for lotions. Such carriers may be present as from about 1% up to about 98% of the formulation. More usually they will form up to about 80% of the formulation.

For administration by inhalation the prodrugs according to the invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, e.g. dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, tetrafluoroethane, heptafluoropropane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of e.g. gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of a compound of the invention and a suitable powder base such as lactose or starch.

The preferred pharmaceutical compositions are those in a form suitable for oral administration, such as tablets and liquids and the like and topical formulations.

The prodrugs may also be administered orally to the patient in a suitable dosage form alone or together with an oral bioavailability-enhancing agent. Such bioavailablity-enhancing agent may be selected from the group consisting of cyclosporins A through Z, (Me-lle-4)-cyclosporin, dihydro cyclosporin A, dihydro cyclosporin C, acetyl cyclosporin A, genistein and related isoflavonoids, quercetin, calphostin, ceramides, morphine and morphine congeners. Preferred enhancing agents are cyclosporin A, cyclosporin C, cyclosporin D, cyclosporin F, dihydro cyclosporin A, dihydro cyclosporin C and acetyl cyclosporin A.

Further, the prodrugs of the present invention may be administered alone or with other chemotherapeutic agents (e.g., other anti-cancer agents), for example: amsacrine, anastrozole, asparaginase, asparaginase monohydrate, azelaic acid, corynebacterium parvum, dacarbazine, docetaxel, docetaxel anhydrous, erwinia asparaginase, etoposide, etoposide phosphate, formestane, gallium, gallium nitrate, mitotane, mitoxantrone, mitoxantrone HCl, paclitaxel, paclitaxel semi-synthetic, pegasparagase, procarbazine, procabazine HCl, razoxane, tamoxifen, tamoxifen citrate, teniposide and topotecan. Preferred chemotherapeutic agents for use in combination therapy treatments with the prodrugs of the invention may be selected from the group consisting of: cisplatin, methotrexate, cytosine arabinoside and toposoimerase inhibitors. Where the prodrugs of the present invention are administered with other chemotherapeutic agents, the prodrugs and other chemotherapeutic agents may be administered simultaneously or sequentially. Additionally, where the drug component drug-oligomer prodrug of the present invention is an anti-cancer agent, such as etoposide, paclitaxel or docetaxel, the prodrug may be administered before, after or simultaneously with radiation therapy.

## 5. Examples

Particular aspects of the present invention will now be described with reference to the following examples. It should be appreciated that these examples are for the purposes of illustrating aspects of the present invention, and do not limit the scope of the invention. The construction of the conjugates of the present invention involved the synthesis of an PEG-oligomer/polymer followed by conjugation to the drug. In the ensuing examples, etoposide is used as the drug; however, it will be appreciated that other drugs having suitable points of attachment (e.g., hydroxyl groups) for the PEG-oligomers/polymers of the present invention may be suitably employed. The applicants have also successfully employed this same general

approach (i.e., conjugation of PEG-oligomer/polymer to hydroxyl groups of a therapeutic compound) using paclitaxel (shown below).

Etoposide contains three reactive hydroxyl groups that can be used to conjugate oligomers (Figure 1). The present invention permits derivatization of all three of these hydroxyl functions. The PEG-oligomer/polymer is attached to the hydroxyl groups on the etoposide molecule via a labile ester bond. The length of the amphiphilic component (e.g., PEG) can be varied to achieve the desired degree of amphiphilicity for the prodrug. The appendage of the PEG-oligomer/polymer not only imparts water solubility, but also behaves like a prodrug, hydrolyzing readily in vivo to release free etoposide.

**5.1 Synthesis of PEG-Oligomer/Polymer-Drug Conjugates**

**5.1.1 Synthesis of Compound I [$ClC(O)CH_2(OC_2H_4)_2OCH_3$]**

In the synthesis of Compound I, 6.2 ml (0.085mol) of thionyl chloride was placed through a dropping funnel into a two-neck flask equipped with a reflux condenser. The flask was heated gently on a water bath and anhydrous [2-(2-Methoxy-ethoxy)-ethoxy]-acetic acid (10g, 0.056mol) was added during the course of 40 min. When all acid had been introduced, the reaction mixture was heated on a water bath for 30 min and reaction mixture was stirred overnight at room temperature. Excess thionyl chloride was removed by aspiration pump and the product was distilled under the reduced pressure. The product was characterized using IR and NMR.

**5.1.2 Synthesis of Amphiphilic Compound II [$ClC(O)CH_2OCH_2CH_2OCH_3$]**

Compound II was prepared using the same general steps as described above in Section 5.1.1 with regard to Compound I with the appropriate polyethylene glycol carboxylic acid starting material.

**5.1.3 Synthesis of Amphiphilic Compound III [$CH_3CH_2OC(O)(CH_2)_2O(CH_2CH_2O)_2CH_2CH_3$]**

In a round bottom flask NaH (3.9g, 0.163mol) was dispersed in 150 ml of anhydrous THF. Anhydrous di (ethylene glycol) ethyl ether (20g, 0.149mol) was dissolved in THF (50 ml) and added dropwise to the NaH suspension. The reaction mixture was stirred for 2 hours. The

solution of ethyl 3-bromopropionate (17.5 ml, 0.140mol) in THF (20ml) was added to a pre-cooled reaction mixture (t =-10°C). The reaction mixture was stirred overnight, and the solvent was evaporated completely. Ethylacetate was added to the residue and a mixture was washed with a solution of 10% NaCl and deionized water. The ethylacetate solution was dried over $MgSO_4$, filtered, and the solvent was evaporated. The residue was dried using a vacuum pump. The crude material was chromatographed on a silica gel column. Fractions containing the Compound III were collected, concentrated and characterized by TLC, HPLC, MS and NMR.

### 5.1.4 Hydrolysis of Compound III to Yield Compound IV

Compound III was stirred with 1N NaOH for 3–4 hours. The reaction mixture was concentrated to remove alcohol and acidified. A solution of NaCl was added and this was extracted with methylene chloride. The organic solution was washed with deionized water, dried over $MgS0_4$, filtered, and the solvent was evaporated to yield Compound IV [$HOC(O)CH_2CH_2(OC_2H_4)_2CH_3$]. The product was characterized by TLC, IR and NMR.

### 5.1.5 Synthesis of Amphiphilic Compound V [$ClC(O)CH_2CH_2(OC_2H_4)_2OCH_2CH_3$]

Compound V was prepared using the same general steps as described above in Section 5.1.1 with regard to Compound III.

### 5.1.6 Conjugation of PEG-Oligomer/Polymer to Etoposide

In a round bottom 3 neck flask 0.250 g (0.4mmol) of etoposide was dissolved in 4 ml of anhydrous chloroform. The solution was stirred under nitrogen atmosphere and 0.32 g (4 mmol) of pyridine was added. Then reaction mixture was cooled by ice bath and [2-(2-Methoxy-ethoxy)-ethoxy]-acetyl chloride was added dropwise. The reaction mixture was stirred for 3 hours then washed with 1% $H_2SO_4$ solution (2x10ml) and deionized water, dried over $MgSO_4$, filtered and concentrated via rotovap.

The crude residue was chromatographed on a silica gel column, using chloroform-methanol 90-10% as a mobile phase. Fractions containing the product were collected, concentrated and dried via vacuum pump.

In initial tests of the etoposide conjugate synthesis scheme, the desired compound (Figure 1) was isolated. The compound was characterized by 1H NMR, MS and HPLC analysis and shown to be the desired product. The solubility of this etoposide conjugate was found to be 7-fold greater than etoposide in phosphate buffer.

**5.2 Cleavage of conjugates in an aqueous environment.**

An example of the hydrolysis of the drug-oligomer conjugate in aqueous solution is set forth in Figure 3. Samples of purified etoposide conjugate in phosphate buffers show increasing rate of hydrolysis as the pH increases. At pH 5.9 approximately 10% of the conjugate was hydrolyzed in 28 hours, while at pH 7.4 approximately 40% was hydrolyzed in the same time period. At pH 8.0 hydrolysis was essentially complete after 20 hours. No significant side products were observed during this conversion.

When the etoposide conjugate was incubated in fresh rat plasma at 37°C hydrolysis of the conjugated etoposide and appearance of free etoposide showed reciprocal behavior (Figure 4). This indicates that hydrolysis of the PEG-oligomer/polymer etoposide conjugate is complete and releases free etoposide. Since free etoposide is known to have anticancer therapeutic effects, as described more fully in Section 1, and since the prodrug created by the applicants is metabolized to active etoposide under normal physiological conditions, it is clear that the prodrug of the present invention is capable of delivering the active anti-cancer drug to a subject.

**5.3 Accumulation of Conjugates in the CNS following I.V. Administration.**

Conjugates of etoposide were administered I.V. to Sprague Dawley rats at 9 μmol/kg. Measurement of free etoposide in plasma (Figure 5) at various time points demonstrated that peak plasma etoposide concentration was increased by 60% and the half-life was extended by approximately 50% in rats given the etoposide conjugate (curve I) versus rats given etoposide (curve II).

When the etoposide concentration in brain tissue was measured in these rats the data revealed that injection with the etoposide conjugate led to a 3-fold increase in accumulation of free etoposide in the brain parenchyma (Figure. 6). These data agree with other studies conducted

by the present inventors demonstrating an increased penetration of the blood-brain barrier by conjugated peptide hormones (data not shown).

**5.4 Confirmation that the Etoposide Conjugate Inhibits Topoisomerase II** Covalent modification of a drug molecule will frequently alter its activity. While it is not strictly necessary that the conjugates do not impede the activity of the parent compound, since the conjugates are hydrolyzed in vivo to provide the active parent compound, it is nevertheless important to assess the biological activity of the etoposide conjugate so that any change in the biological activity can be factored into interpretation of data from subsequent experiments.

The ability of the etoposide conjugate to inhibit topoisomerase II activity can be assessed using a DNA-unknotting assay (Isabella, Capranico et al. 1990). In this assay, topoisomerase II resolves high molecular weight, highly interwound phage P4 DNA into smaller DNA fragments. Briefly, highly knotted P4 DNA is prepared and incubated with high salt nuclear extracts from HeLa cells containing topoisomerase II as described (De Isabella, Capranico et al. 1990; Giaccone, Gazdar et al. 1992). Varying concentrations of etoposide conjugate or native etoposide can be added to the reactions. After a 30-minute incubation at 37°C, the reactions are terminated by addition of sodium dodecyl sulfate and proteinase K and the DNA fractionated by gel electrophoresis. High and low molecular weight DNA can be visualized by ethidium bromide staining.

**5.5 Further Definition of the Biodistribution and Pharmacokinetics of the Etoposide Conjugate**

The focus of the present effort is to enable active etoposide to cross physiologic barriers that free etoposide penetrates poorly. Recently published studies indicate that the P-glycoprotein multidrug resistance molecule and related proteins may be a key component of the barrier to etoposide penetration in the brain parenchyma. The drug transport molecules may also be responsible for the erratic intestinal absorption of etoposide.

Protein Delivery's conjugation technology enables more effective distribution of the conjugated molecule across both of these barriers. The amount of the etoposide conjugate that reaches various tissues, including the brain parenchyma and tumor tissue implanted in the brain, following I.V. administration can be measured. It can then be determined whether oral

dosing with the etoposide conjugate can also result in therapeutic levels of free etoposide in the above tissues.

A number of studies suggest that extended exposure to etoposide may improve its cell-killing ability. In vivo etoposide has a relatively short half-life, which in turn requires repeated administration and attendant medical support. A particularly useful aspect of the conjugation technology is that the rate of hydrolysis of the prodrug can be adjusted to extend the half-life of etoposide in plasma. This offers the possibility to more readily titrate maintenance doses of the conjugate and more easily maintain plasma levels of free etoposide in the therapeutic range.

***Animals and the 9L glioma tumor model.*** Adult Fischer rats (5–6 weeks of age) can be used for these studies. Experimental gliomas can be induced in the rats by stereotactic injection of 20,000 9L glioblastoma cells (Asai, Shibui et al. 1990) from in vitro cultures into the right frontal lobe. After 14 days the animals can be used for experiments.

***Biodistribution following I.V. administration.*** Fischer rats (approximately 250g weight) can be administered etoposide (5 mg/ml in dimethyl sulfoxide/water) at a dose of 5 mg/kg or an equivalent molar mass of the etoposide conjugate in phosphate buffered saline and injected through the caudal vein. Rats can be sampled at 4 hours and 24 hours. Groups of six rats from each treatment group can be euthanized by pentobarbital overdose before plasma and tissue harvest.

Blood samples can be collected by cardiac puncture into heparinized syringes. The blood samples can be centrifuged and the plasma stored at -20°C until analysis. Tissues (heart, lung, brain, kidney, liver, skeletal muscle and tumor) can be rapidly dissected, rinsed in cold phosphate buffered saline, then snap frozen in liquid nitrogen until further processing.

***Biodistribution Following Oral Administration.*** A similar experimental design can be followed as for the oral dosing except that the drugs can be administered by oral gavage using a 20 ga. gavage needle. Etoposide can be diluted to 5 mg/mL in saline for a dose volume of 100μL; etoposide conjugates can be diluted to yield a similar molar mass dose in 100μL. Tissues can be harvested at the same time points and process the same way.

***Pharmacokinetics following oral and I.V. dosing.*** Tumor-bearing Fischer rats can be administered etoposide or an equivalent molar mass of the etoposide conjugate as described for the biodistribution studies except that two dose levels can be tested: 1 mg/kg and 5 mg/kg. Groups of six rats can be sampled at 5, 10, 15, 30 and 60 minutes, as well as 2 hours, 4 hours and 24 hours. The rats can be euthanized by phenobarbital overdose and blood samples can be collected by cardiac puncture into heparanized syringes. Cells in the blood samples can be pelleted by brief centrifugation and plasma stored at -20°C.

***Analysis of etoposide in plasma and tissues.*** Plasma and tissue concentrations of etoposide can be measured by HPLC (Eiseman, Eddington et al. 1994). Samples of plasma can be mixed 1:2 with acetonitrile. The samples can then be acidified by addition of an equal volume of 0.02 M sodium acetate (pH 4) to prevent hydrolysis of etoposide conjugates in the sample. Tissues can be thawed and immediately homogenized in two volumes of saline. One volume of homogenate can be mixed with 2 volumes of acetonitrile, then acidified by addition of and equal volume of sodium acetate as above. Cell debris in all of the samples can be removed by brief centrifugation.

***Data analysis.*** Etoposide concentration in each sample can be determined by calculating the ratio of the area under the etoposide peak to that of the internal control. The concentration in mg/ml can be determined by comparison to a previously constructed standard curve for the internal control. Data can be tabulated as the mean [etoposide] +/-SE.

***Pharmacokinetic analysis.*** Drug concentration-plasma profiles can be analyzed by using R-strip software. The area under the plasma concentration-time curve can be calculated and extrapolated to infinity. Bioavailability following oral administration can be calculated using the formula %=(AUCoral/AUCi.v.) x 100. The total body clearance can be calculated using the equation CLtb = Dose/AUC and the volume of distribution can be calculated from the formula Vd = CLtb/Kel. The half life of distribution and half life of elimination can be determined by extrapolation from the initial and secondary log linear portions of the plasma-time concentration curves.

**5.6 Confirmation of Efficacy of the Etoposide Conjugate**

Conjugation of etoposide to PEG-oligomers/polyrners is expected to enable treatment of CNS tumors through parenteral administration and potentially by oral administration as well. The

etoposide conjugate can be tested for its ability to extend the life expectancy of rats bearing 9L tumors intracranially.

***Tumor models.*** The 9L cell line can be maintained in culture as recommended by the American Type Culture Collection. Tumors can be induced by stereotactic inoculation of cells into the right frontal lobe of Fischer rats as described above. Rats can be used 14 days after intracranial inoculation. Prior to treatment, rats can be pooled and randomized into treatment groups.

***Treatment regimen.*** The treatment regimen can use the highest non-lethal dose of etoposide or the etoposide conjugate as determined from the toxicity experiments. Groups of 10 rats can be treated with each compound and 10 can serve as untreated controls. Rats can be weighed on the first day of treatment and every other day thereafter.

Rats can be treated on days 1, 3, 5 and 7. The dose of drug can be given orally and I.V. for the glioblastomas. Neurological signs including crouching, lethargy and paralysis can be the endpoint for rats bearing glioblastomas. Rats can be observed for neurological signs daily. If death occurs between observations this can be used as the endpoint.

Samples can be analyzed on a Waters Alliance 2690 system using a water/acetonitrile mobile phase on a C-18 column with U.V. detection at 230nm. Concentrations of analyte can be determined by comparing the area under the curve on the HPLC trace to a standard curve. Tissue and plasma samples can be spiked with 25 (g/mL cephalomannine as an intemal control to correct for extraction efficiency.

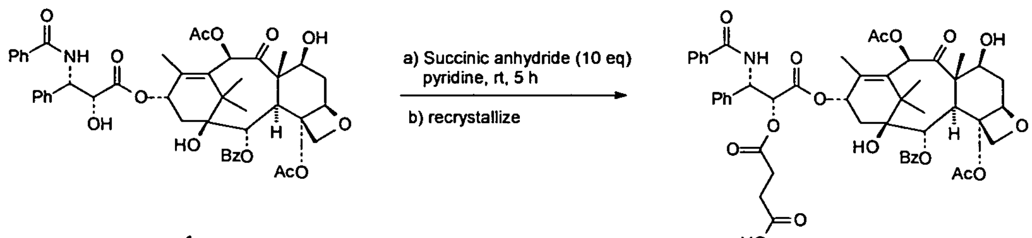
**5.7 Determination of Optimum Dose of Conjugated Etoposide**

The optimum dose of conjugated etoposide can be determined in animal models. Extensive preclinical efficacy testing can be performed using multiple tumor models, both for CNS tumors and non-CNS tumors.

It is expected that the above-described empirical procedures will confirm an increase in free or conjugated etoposide concentration in brain parenchyma of animals given the etoposide conjugate relative to controls given etoposide. It is further expected that this work will confirm a statistically significant effect on survival of tumor-bearing animals given the

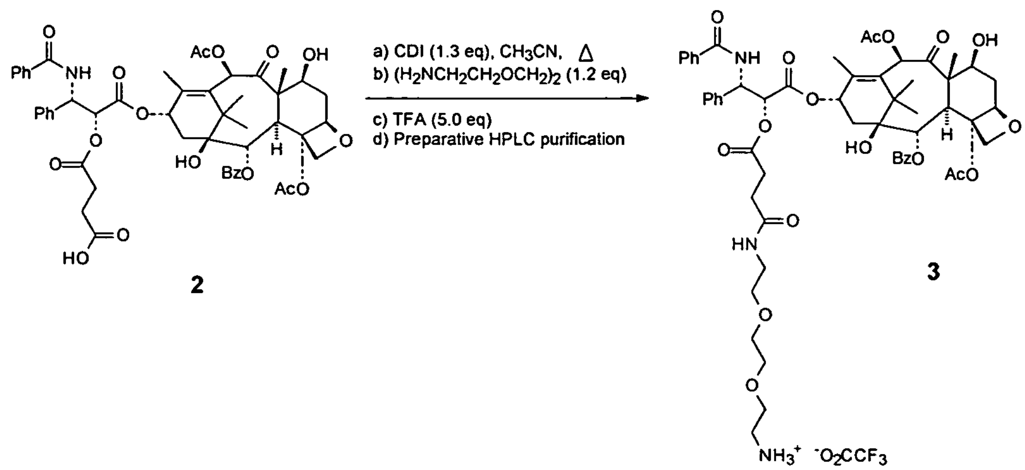
conjugate relative to untreated controls. Additionally, oral bioavailability will be significantly increased in animals given the conjugate relative to animals given etoposide.

### 6.1 2'-Succinylpaclitaxel




The following protocol is an improved version of the procedure of Deutsch, *et. al.* To a mixture of 5.00 g (5.86 mmol) of paclitaxel and 5.86 g (58.6 mmol) of succinic anhydride was added 110 mL of anhydrous pyridine. After stirring the resulting solution at room temperature for 5 h, thin layer chromatography (TLC) analysis indicated complete consumption of the paclitaxel. The solvent was removed under reduced pressure by means of rotary evaporation and the residue was dried *in vacuo* for 2 h. The resulting waxy semisolid was stirred efficiently with 200 mL of water, affording a flocculent white solid, which was collected by suction filtration on a Buchner funnel. The solid was washed with water then allowed to suck dry for 30 mm., then dried at room temperature *in vacuo* in a dessicator over $P_20_5$ for 15 h. The white solid was taken up in 30 mL of acetone, then with efficient stirring, 30 ml of water was slowly added. The resulting thick white paste was well-blended for 15 mm, suction filtered through a Buchner funnel rinsing with excess water, and allowed to suck dry for 1 h. The resulting moist white solid was carefully dried at room temperature *in vacuo* in a dessicator over $P_2O_5$ for 18 h. As the solid became more dry and less coagulated during this drying period, the $P_2O_5$ was replenished and the solid was periodically broken into smaller pieces until a moderately fine powder was obtained (5.29 g, 95% yield). Analytical HPLC analysis indicated this material to be of 97% purity. MS (FAB+) *m/z* (rel. inten.) 954 ($M^+$, 100), 570 (15), 509 (64).

## 6.2 2'-Succinamidyl-PEG2-amine-paclitaxel trifluoroacetate



A mixture of 200 mg (0.2 10 mmol) of 2'-succinylpaclitaxel (Compound 2) and 47 mg (0.273 mmol, *95%* purity) of 1,1 '-carbonyldiimidazole under nitrogen was dissolved in 2.9 mL of anhydrous acetonitrile. A gas outlet needle was inserted through the reaction flask septum such that a moderate flow of nitrogen was maintained to flush out carbon dioxide gas. The reaction vessel was lowered into an oil bath preheated to 48°C, causing gas evolution. After stirring efficiently for 15 min, the reaction vessel was removed from the oil bath and allowed to cool to room temperature. The amount of acetonitrile lost to evaporation was replenished. The nitrogen outlet needle was removed such that a static atmosphere of nitrogen was maintained. A solution of 37 µL (0.252 mmol) of 2,2'-(ethylenedioxy)bis(ethylamine) in 1.4 mL of acetonitrile was added dropwise. After 45 min., a solution of 81 µL (1.05 mmol) of trifluoroacetic acid in 0.7 mL of acetonitrile was added dropwise. The resulting crude reaction solution typically contains a 70-74% yield of the desired trifluoroacetate product (Compound 3), as determined by analytical HPLC analysis.

## 6.3 Preparative HPLC Purification of Trifluoroacetate (Compound 3)

The product was generally purified by prep HPLC by combining the product-containing fractions.

### 6.3.1 Improving HPLC Resolution

**Medium Scale Reaction.** To improve HPLC resolution on a medium scale, the crude reaction solution was diluted by adding water without causing precipitation. Thus, to an aliquot of 3.30 mL of the above reaction solution was slowly added 4.03 mL of water with efficient stirring, consequently affording a solution consisting of 55% water. The resulting slightly hazy mixture was filtered through a 0.45 μm Gelman acrodisc 13 syringe filter, then chromatographed on a Waters 600E HPLC system using a reverse phase Vydac column (22 mm x 250 mm, C 18, 300Å, 10-15μ). The mobile phase was an acetonitrile-water solution containing 0.1% (v/v) trifluoroacetic acid. Major fractions containing desired product of >97% purity were obtained using a gradient elution, from 40:60 to 45:55, acetonitrile:water with 0.1% (v/v) TFA over 30 minutes at a flow rate of 5 mL/mm. Subsequent isocratic elution with 90:10, acetonitrile:water containing 0.1% (v/v) TFA removed unidentified side products in preparation for subsequent chromatographic runs.

For analytical purposes, the purified fractions resulting from the above-described amidation reaction protocol [200 mg (0.210 mmol) scale of 2'-succinylpaclitaxel (2)] were concentrated under reduced pressure by means of rotary evaporation with gradual bath warming to 55°C, then dried in vacuo at room temperature to obtain 170 mg (68% yield) of trifluoroacetate (Compound 3) as an amorphous white solid. $^1$H NMR (300 MHz, $CDCl_3$) δ 8.10 (2H, d, J=7.2 Hz), 7.99-7.81 (2H, m), 7.66-7.20 (11H, m), 6.92 (1H, brs), 6.28 (1H, s), 6.07 (1H, t, J=10.0 Hz), 5.86 (1H, dd, J=3.9, 5.0 Hz), 5.65 (1H, d, J=6.9 Hz), 5.42 (1H, d, J=5.4 Hz), 4.94 (1H, d, J=8.2 Hz), 4.36 (1H, m), 4.29 (1H, d, J=8.5 Hz), 4.16 (1H, d, J=8.5 Hz), 3.74 (1H, d, J=6.9 Hz), 3.66-3.04 (16H, m), 2.80-2.60 (2H, in), 2.38 (3H, s), 2.20 (3H, s), 1.84 (3H, s), 1.66 (3H, s), 1.20 (3H, s), 1.13 (3H, s); MS (FAB+) *m/z* 1084 ($M^+$).

**Larger Scale Reaction.** A larger scale amidation reaction solution consisting of 1,000 mg (1.05 mmol) of 2'-succinylpaclitaxel (Compound 2), 27 mL total volume was shown to contain 44% of desired trifluoroacetate product (Compound 3) by analytical HPLC analysis. A 10.0 mL aliquot was concentrated under reduced pressure by means of rotary evaporation just to the point of affording a slightly viscous yellow oil. The oil was dissolved by adding 1.0 mL of acetonitrile, filtered through a 0.45 μm Gelman acrodisc 13 syringe filter, then chromatographed on a Waters 600E HPLC system using a reverse phase Vydac column (50 mm x 250 mm, C18, 300Å, 10-15μ). The mobile phase was an acetonitrile-water solution

containing 0.1 % (v/v) trifluoroacetic acid. Major fractions containing desired product of >97% purity were obtained using a gradient elution, from 40:60 to 45:55, acetonitrile:water with 0.1% (v/v) TFA over 30 minutes at a flow rate of 26 mL/min. Subsequent isocratic elution with 90:10, acetonitrile:water containing 0.1% (v/v) TFA removed unidentified side products in preparation for subsequent chromatographic runs.

The fractions determined by analytical HPLC analysis to be of high purity were combined with analogous preparative HPLC runs to provide a combined solution of 870 mL total volume containing trifluoroacetate (Compound 3) of >97% purity. The solution was used directly (without concentration) in the ion exchange chromatography step.

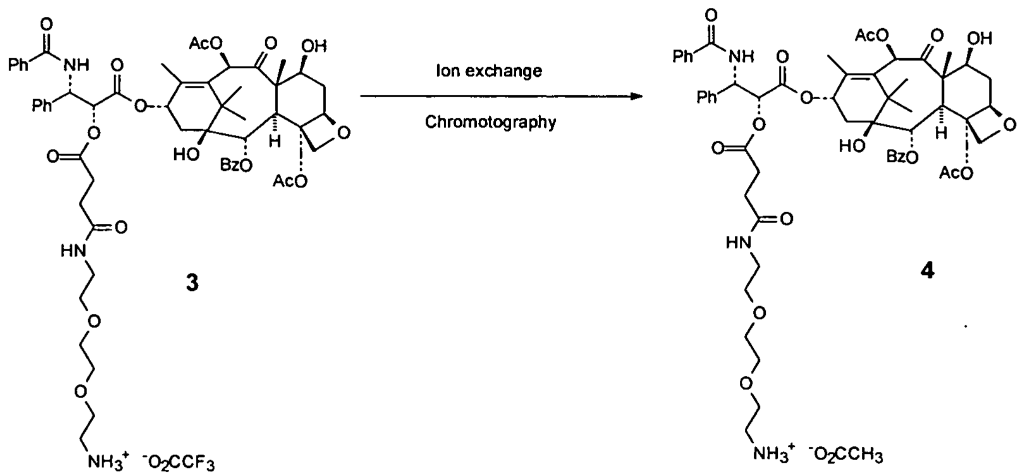
### 6.4 Stability of Trifluoroacetate (Compound 3)

It was observed that several trifluoroacetate product fraction solutions obtained from a preparative HPLC run initially of an average purity of 98.8% changed to an average of 97.4% purity after storage at $8^{0}$C for 16 days, as determined by analytical HPLC analysis.

#### 6.4.1 Determination of Aqueous Solubility of Trifluoroacetate (Compound 3)

To a sample of 2.5 mg of trifluoroacetate (Compound 3) in a small vial was added 200 μL of deionized water. The vial was capped and the resulting mixture was ultrasonicated for 15 minutes. The resulting cloudy mixture was filtered through a 0.45 μ Gelman acrodisc 13 syringe filter. The filtrate was weighed (140 mg) and was lyophilized to provide 1.4 mg of a white fluffy solid. Thus, assuming that the aqueous solution is of density 1.00, the water solubility of trifluoroacetate (Compound 3) is 7.4 mg/mL (i.e. 1.4 mg/0.190 mL).

## 6.5 Ion Exchange Chromatography of Trifluoroacetate Anion for Acetate Anion: Acetate Prodrug (Compound 4)



For preparative purposes, fraction solutions of trifluoroacetate (Compound 3) of >97% purity obtained from a preparative HPLC purification protocol described above were combined and taken on directly to ion chromatography without concentration. For example, combined preparative HPLC fractions of 870 mL total volume theoretically containing 0.462 mmol of substrate and containing 11.3 mmol of TFA (0.1 % (v/v)) was used for the following ion exchange protocol:

A 92 g portion of DOWEX® 1 x 8-400 (strongly basic, chloride form) ion exchange resin was washed three times with 270 mL each of deionized water, each time decanting away the yellow suspended matter and the bulk of the rinse water. To obtain the resin in its acetate form, the resulting slurry was stirred with a solution of 1,882 g of $NaOAc{\cdot}3H_20$ in 4.00 L of deionized water for 1.0 h. The resin was collected by suction filtration on a Buchner funnel and washed several times with a total of 1.84 L of deionized water. The resin was washed two times with 750 mL each of 0.013 M HOAc (aq) [i.e. 0.1% (v/v) HOAc, by analogy to 0.1% (v/v) TFA used in the preparative HPLC protocols], suction filtered on a Buchner funnel, then allowed to suck dry for 10 min. The resin was suspended in 0.013 M HOAc (aq), poured into a glass flash chromatography column, then eluted using gentle air pressure with 200 mL of 0.013 M HOAc (aq), thus giving a 10 cm high x 3.8 cm diameter resin column, which was then topped off with *ca.* 4 cm of sand. The column was then eluted with 250 mL of acetonitrile, then with 250 mL of 0.0 13 M HOAc (aq). The 870 mL volume of

the trifluoroacetate (3) solution was made to be 0.0 13 M in HOAc by the addition of a solution of 0.63 mL of glacial acetic acid in 10 mL of acetonitrile with efficient stirring. The solution was applied to and eluted through the column using gentle air pressure. Fraction collection was commenced immediately using 50 mL test tubes. Once the entire 870 mL of solution had completely passed into/through the resin, the column was further eluted with 0.013 M HOAc (aq). The fractions containing product of >97% purity (as determined by analytical HPLC analysis at 210 nm) were combined and concentrated under reduced pressure by means of rotary evaporation with gradual bath warming to 55°C and dried *in vacuo* at room temperature for 16 h to provide an off-white amorphous residue. The residue was scraped with a spatula from the flask into a fine amorphous powder. The remnants not scraped out of the flask could be further procured without alteration of product purity by transferring to a smaller flask with the aid of a minimal amount of 0.013 M HOAc (aq) as solvent, concentrating as described above, then scraping with a spatula to provide additional product, which was combined and dried at room temperature *in vacuo* in a dessicator over $P_2O_5$ for 24 h to provide acetate prodrug (Compound 4) (293 mg, 24% unoptimized yield overall of material of >97% purity) as a yellow powder. Combination of the fractions of <97% purity in a manner similar to the method described above afforded 146 mg (12% unoptimized yield) of additional acetate (Compound 4). Analyses of the products by analytical ion chromatography [Quantitative Technologies, Inc., (QTI)] did not show the presence of any trifluoroacetic acid (TFA analysis: below detection limit of 100 ppm). mp 114-117°C; $^1$H NMR (300 MHz, $CDCl_3$) δ 8.11(2H, d, J=7.2 Hz), 7.85 (2H, d, J=7.7 Hz), 7.62-7.36 (11H, m), 6.29 (1H, s), 6.11 (1H, t, J=10.0 Hz), 5.87 (1H, dd, J=3.9, 4.4 Hz), 5.65 (1H, d, J=6.9 Hz), 5.44 (1H, d, J=4.9 Hz), 4.95 (1H, d, J=9.5 Hz), 4.38 (1H, m), 4.28 (1H, d, J=8.5 Hz), 4.18 (1H, d, J=8.2 Hz), 3.76 (1H, d, J=6.4 Hz), 3.63-3.34 (16H, m), 3.04 (2H, brs), 2.75 (2H, in), 2.53 (2H, m), 2.40 (3H, s), 2.20 (3H, s), 2.01 (3H, s), 1.87 (3H, s), 1.67 (3H, s), 1.20 (3H, s), 1.13 (3H, s); MS (FAB+) *m/z* (rel. inten.) 1084 ($M^+$).

**6.6 General Solubility of Acetate (Compound 4)**

Acetate (Compound 4) is highly soluble in $CDCl_3$ and sparingly soluble in diethyl ether and in hexanes. Three separate samples of 3.0 mg each of acetate (Compound 4) afforded a homogeneous solution in 81 μL of 100% anhydrous ethanol, in 81 μL of acetone, and in 81 μL of 0.5 M aqueous acetic acid. Thus, the solubility in each of these three solvents must be some value equal to or greater than 37 mg/mL (i.e. 3.0 mg/ 0.08 1 mL).

### 6.7 Determination of Aqueous Solubility of Acetate (Compound 4) by Mass

To a sample of 10.0 mg of acetate (Compound 4) in a small vial was added 176 μL of deionized water. The vial was capped and the resulting mixture was ultrasonicated for 15 minutes. The resulting liquid was shown to contain acetate (Compound 4) in 98.7% purity by analytical HPLC analysis at 210 nm. The liquid was filtered through a 0.45 μ cellulose acetate syringe filter. The mass (76mg) of a known volume (75 μL) of the filtrate was measured to determine that the density of the solution was 1.01 g/mL. The filtrate was shown to contain acetate (Compound 4) in 98.8% purity by analytical HPLC analysis at 210 nm. A 118 mg quantity of the filtrate was lyophilized to provide 6.4 mg of an off-white fluffy solid. Thus, by this method the water solubility of acetate (Compound 4) was determined to be 55 mg/mL (i.e. 6.4 mg/0.117 mL).

### 6.8 Determination of Aqueous Solubility of Acetate (Compound 4) by Analytical HPLC

To a sample of 10.0 mg of acetate (Compound 4) in a small vial was added 150 μL of deionized water. The vial was capped, vortexed for 3 mm., and ultrasonicated for 15 minutes. The resulting viscous pale yellow/orange mixture was slowly and carefully taken up into a 1 mL syringe and then firmly filtered through a 0.45 μ cellulose acetate syringe filter, thus affording a viscous homogeneous yellow/orange solution. A 75 μL portion of the filtrate was diluted 88-fold with acetonitrile and then analyzed by analytical HPLC at 229 nm and 270 nm. The area under the curve observed at 229 nm and 270 nm was respectively compared to both a 3-point calibration curve obtained at 229 nm ($r^2 = 0.99999$) and a 5-point curve obtained at 270 nm ($r^2 = 0.99996$). Thus, by this method, the water solubility of acetate (Compound 4) was determined to be 40.1 mg/mL (at 229 nm) and 39.7 mg/mL (at 270 nm).

### 6.9 Chemical Hydrolysis Behavior of Acetate (Compound 4)

A sample of solid acetate (Compound 4) was dissolved *(ca.* 1 mg/mL) in wet acetonitrile (nonanhydrous due to exposure to humid air). Over the course of 26 days at room temperature, acetate (Compound 4) smoothly converted quantitatively into paclitaxel (1) with no significant side product formation, as observed by analytical HPLC analysis (see Figure 1).

### 6.9.1 Chemical Hydrolysis Behavior of Acetate (Compound 4) at Various pH

To many 13 mm x 100 mm glass test tubes was added 5.15 μL each of an $8.741 \times 10^{-4}$ M aqueous solution of acetate prodrug (Compound 4). To each tube was then added 295 μL of aqueous solutions of phosphate buffered saline (PBS) of pH 8.00, 7.40, 7.00 and 5.80 and an acetic acid/formic acid buffered solution of pH 2.00. The tubes were capped and incubated at 37°C in a reciprocal water bath shaker. After incubation for various appropriate time intervals, a test tube was removed from the shaker, 900 μL of acetonitrile was added, and the sample was vigorously vortexed for 3 minutes. The resulting solutions were analyzed by analytical HPLC to generate hydrolysis rate data (see table and graphs, below).

### 6.10 *In Vitro* Prodrug Hydrolysis of Acetate (Compound 4)

To nine 13 mm x 100 mm test tubes was added 5.15 μL each of an $8.741 \times 10^{-4}$ M aqueous solution of acetate prodrug (Compound 4). To each tube was then added 295 μL of freshly obtained heparinized rat plasma from an adult male Sprague Dawley rat (CD) (source Charles River; Raleigh, NC). The tubes were capped and incubated at 37°C in a reciprocal water bath shaker. After incubation for various time intervals, a test tube was removed from the shaker, 900 μL of acetonitrile was added, the sample was vigorously vortexed for 3 minutes and then cooled to -12°C for between 30 and 90 minutes. The samples were centrifuged at 25°C for 10 minutes at 1,600 g and the resulting clear colorless supernatant solution was analyzed by analytical HPLC (see Figure 2).

### 6.11 2'-Succinamidyl-PEG2-amine-paclitaxel acetate

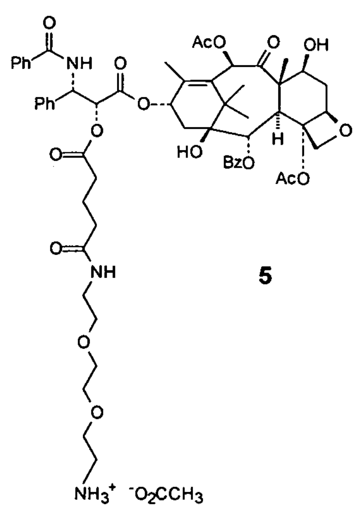
A mixture of 200 mg (0.210 mmol) of 2'-succinylpaclitaxel (Compound 2) and 47 mg (0.273 mmol) of 1,1'-carbonyldiimidazole under nitrogen was dissolved in 5 mL of anhydrous acetonitrile. The reaction vessel was lowered into an oil bath preheated to 48°C, causing gas evolution. After stirring for 15 min, the reaction vessel was allowed to cool to room temperature. A solution of 37 μL (0.252 mmol) of 2,2'-(ethylenedioxy)bis-(ethylamine) in 2 mL of acetonitrile was added dropwise, then after 1 h a solution of acetic acid (1.05 mmol) in 1 mL of acetonitrile was added. The resulting crude reaction solution obtained by this method typically contains a 75% yield of desired acetate product, as determined by analytical HPLC analysis.

**6.12 Preparative HPLC Purification of 2'-Succinamidyl-PEG2-amine-paclitaxel acetate**

The crude compound from Example 5.11 was chromatographed on a Waters Prep LC 4000 system, using a reverse phase Vydac column (22 mm x250 mm, C18, 300Å, 10-15μ). The product was purified by combining the product-containing fractions obtaining from preparative HPLC. Major fractions containing desired product (>97% purity) were obtained using a gradient elution, from 30:70 to 95:5, acetonitrile:water with 0.1%(v/v) acetic acid over 35 min. The purified fraction were concentrated under reduced pressure by means of rotary evaporation, then lyophilized to obtain white solid. MS (FAB+) m/z 1084 ($M^+$).

**6.13 2'-Glutaramidyl-PEG2-amine-paclitaxel acetate**

2'-Glutarylpaclitaxel is synthesized using the procedure described above in Example 5.1 by substituting glutaric anhydride for succinic anhydride. 2'-Glutaramidyl-PEG2-amine-paclitaxel acetate (Compound 5) is then synthesized using the procedure described above in Example 5.11 by using 2'-glutarylpaclitaxel instead of 2'-succinylpaclitaxel as a starting material. The chemical structure of compound 5 is shown below.



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**6.14 Preparative HPLC Purification of 2'-Glutaramidyl-PEG2-amine-paclitaxel acetate**

Preparative HPLC purification of 2'-glutaramidyl-PEG2-amine-paclitaxel acetate is performed using the procedure described above in Example 5.12.

### 6.15 Effect of Compounds of the Present Invention on the NIH:OVCAR-3 Cell Line

The purpose of this experiment was to investigate the effect of a full dose response of Paclitaxel (tested for comparison purposes only, not a compound of the present invention), TX-001 (Compound 4, described above in Example 5.5), and TX-002 (Compound 5, described above in Example 5.13) on the NIH:OVCAR-3 cell line. NIH:OVCAR-3 cells, which are a human ovarian cancer cell line, were removed from a T75 flask by versene and counted via a hemacytometer. The cells were then pelleted by centrifugation (1000 RPM for 10 minutes). The supernatant was removed and fresh media (RPMI 1640 w/ 10%FCS and 1% L-glutamine) was added to bring cell concentration to 10,000 cells/mL. Then 0.1 mL (1000 cells) of the 10,000 cell/mL solution was added to individual wells of a 96 welled flat bottom plate. This plate was then placed overnight in a 37°C with 5% $CO_2$ incubator to allow cells time to adhere to bottom of wells. The next day Paclitaxel, TX-001, and TX-002 were diluted in fresh media to the following concentrations: 1000, 500, 200, 50, 10, 5, 1 and 0.5 nM. Next 0.1 mL of the appropriate drug concentration was added to appropriate column of 96 welled plate (n=8) making final concentrations of 500, 250, 50, 25, 5, 2.5, 0.5, and 0.25 nM. One column on each plate received 0.1 mL of fresh media and served as the untreated control. These plates were then placed in the 37°C with 5% $CO_2$ incubator for 6 days. After 6 day incubation, all wells were aspirated and plates were placed in a -70°C freezer for 2 hours. Also an appropriate number of cells were removed from another T75 flask in the same fashion as mentioned above but this time after centrifugation the supernatant was removed and the tube was placed in a -70°C freezer for 2 hours. The plates and tube were thawed at room temperature and then the CyQuant assay (Molecular Probes) was performed. The tube was resuspended with an appropriate amount of the CyQuant GR dye/cell lysis buffer and diluted accordingly to create a standard curve. Next 0.2 mL of each standard concentration was placed in duplicate in a new 96 well plate. This plate was then placed in the dark for 3 minutes. Finally all wells of the 96 well plates that were previously frozen and thawed received 0.2 mL of CyQuant GR dye/cell lysis buffer and as well were placed in dark for 3 minutes. The fluorescence was then measured at 485 nm excitation and 538 nm emission. Cell number per each well was then calculated from the standard curve. Means were calculated for each concentration of drug and then were divided by the mean of the untreated wells for each particular plate. The results are shown in Figure 3 and expressed in % growth to untreated cells.

### 6.16 Effect of Compounds of the Present Invention on the Production of Activated Caspase-3 by the NIH:OVCAR-3 Cell Line

The purpose of this experiment was to investigate the effect of 50 nM Paclitaxel, TX-001, and TX-002 on the production of activated Caspase-3 by the NIH:OVCAR-3 cell line. 7 T75 flasks with approximately 70-80% NIH:OVCAR-3 cell confluence were used for this experiment. Each flask was rinsed with 5 mL of PBS then 15.0 mL of appropriate drug were added to each flask. These flasks were then placed in a 37°C with 5% $CO_2$ incubator until desired time point was achieved. Each T75 flask was then 0.05% Trypsin treated and cells were counted via a hemacytometer. The cells were then pelleted by centrifugation (1000 RPM for 10 minutes). The amount of active Caspase-3 was then determined by the Caspase-3 Fluorometric Assay (R&D Systems). The supernatant was removed from the pelleted cells and 25 μL of cold Cell Lysis Buffer is added for every 1,000,000 cells/mL. This cell lysate was incubated on ice for 10 minutes and then 50 μL from each condition is added in duplicate to a 96 well flat bottom plate. Next 50 μL of 2X Reaction Buffer and 5 μL of Caspase-3 fluorogenic substrate (DEVD-AFC) were added to each well. The plate was then placed in a 37°C with 5% $CO_2$ incubator for 1.5 hours. Finally the fluorescence was then measured at 400 nm excitation and 505 nm emission. Data was then plotted as active Caspase-3 Fluorescence as illustrated in Figure 4.

### 6.17 Evaluation of the Anti-tumor Activity of Compounds 4 and 5 Against a Series of Human Tumor Cell Lines *In Vitro*

HT-1080 and HT-1080/DR4 (MRP/LRP positive) fibrosarcoma cell lines (1,2) were obtained from Dr. Y. Rustum, Roswell Park Cancer Institute, Buffalo, NY. The A121 ovarian cell line (3) was provided by Dr. Kent Crickard, Buffalo General Hospital, Buffalo, NY. The MDA435/LCC6-WT and MDR1 transfected cell lines (4) were provided by Dr. R. Clarke, Lombardi Cancer Center, Georgetown University School of Medicine. Cell lines were propagated as monolayers in RPMI-1640 containing 5% FCS, 5% NuSerum IV, 20 mM HEPES, 2 mM L-glutamine at 37°C in a 5% $CO_2$ humidified atmosphere.

Paclitaxel, which was used for comparison purposes and is not a compound of the present invention, was provided by Dr. I. Ojima, SUNY at Stonybrook. TX-001 (Compound 4) and TX-002 (Compound 5) (CD# 20027 and 20028 respectively) were provided by NOBEX.

Paclitaxel was solubilized in 100 % DMSO. TX-001 and 002 were solubilized in sterile $H_20$ acidified with 10% 1 N HCl. For the first experiment, 4.0 mM stocks of TX-001 and 002 were prepared. Solutions of TX-001 and TX-002 contained clear needle-like and cast-like precipitates and were fairly viscous (Experiment 1). For the second experiment (Experiment 2), less concentrated stock solutions (0.4 mM) of each compound were prepared in sterile $H_20$ acidified with 10% 1 N HCl. The same type of precipitates were noted, however neither solution was viscous. All compounds were further diluted in RPMI-1640 containing 10 mM HEPES.

Assessment of cell growth inhibition was determined in 96 well microtiter plates according to the method of Skehan et al., "New Colorimetric Cytotoxicity Assay for Anticancer-Drug Screening, *Articles* 82(13):1107-1112 (1990). Briefly, cells were plated with 400-1800 cells/well in 96 well plates and incubated at 37°C 15-18 hr prior to drug addition to allow for cell attachment. Each cell line was exposed to 12 concentrations of compound (a 6 log range), 5 replicate data points per compound concentration. Cells were exposed to compound concentrations ranging from 0.003 to 1000 nM for paclitaxel and 0.03 to 10000 nM for TX-001 and TX-002. Cells were cultured for a total of 3-4 cell doublings (72-96 h). Assays were terminated with 100 μl of ice-cold 50% TCA. After 1 hr at 4°C., plates were washed 5 times with tap water to remove TCA, low-molecular-weight metabolites and serum proteins. 50 μl of 0.4% sulforhodamine B (SRB) was added to each well. Following a five minute incubation at room temperature, plates were rinsed 5 times with 0.1% acetic acid and air dried. Bound dye was solubilized with 10 mM Tris Base (pH 10.5) for 5 min on a gyratory shaker. Optical density was measured at 570 nm.

Data were fit with the Sigmoid-Emax concentration-effect model (N.H.G. Holford and L.B. Scheiner, "Understanding the dose-effect relationship: Clinical applications of pharmacokinetic-pharmacodynamic models," *Clin. Pharmacokin.* 6:429-453 (1981)) with nonlinear regression, weighted by the reciprocal of the square of the predicted response. The fitting software was developed at RPCI with Microsoft FORTRAN, and uses Marquardt algorithm (D.W. Marquardt, "An algorithm for least squares estimation of nonlinear parameters," J. Soc. Ind. Appl. Math. 11:431-441) as adapted by Nash (J.C. Nash, <u>Compact Numerical Method for Computers: Linear Algebra and Function Minimization</u>, New York:

John Wiley & Sons, 1979) for the nonlinear regression. The concentration of drug that resulted in 50% growth inhibition ($IC_{50}$) was calculated and is shown in Table I below.

**Table I**
**Effect of Paclitaxel, TX-001 and TX-002 on Human Tumor Cell Growth**

| Compound | Experiment | IC50 nM (±S.E.)[1] | | | | |
|---|---|---|---|---|---|---|
| | | *A121* | *LCC6-WT* | *LCC6-MDR* | *HT-1080* | *HT-1080 -DR4* |
| **Paclitaxel** | 1 | 11 ±0.5 | 4.7 ±0.5 | 434 ±28 | 4.5 ±0.2 | 4.9 ±0.4 |
| | 2 | 14 ±1.3 | 6.0 ±2.0 | 391 ±52 | 5.7 ±0.3 | 7.7 ±0.1 |
| **TX-001** | 1 | 13 ±0.5 | 4.9 ±0.5 | 337 ±20 | 4.5 ±0.4 | 8.9 ±0.4 |
| | | | 2.7±0.4[2] | 269 ±31[2] | | |
| | 2 | 37 ±1.2 | 13 ±0.5 | 404 ±63 | 19 ±0.7 | 36 ±1.5 |
| **TX-002** | 1 | 48 ±3.0 | 18 ±1.1 | 1073 ±45 | 22 ±0.9 | 32 ±1.5 |
| | | | 16 ±2.0[2] | 879 ±78[2] | | |
| | 2 | 171 ±8.8 | 38 ±4.6 | 1798 ±168 | 49 ±6.5 | 54 ±5.3 |

[1]Human tumor cell lines: A121 - ovarian carcinoma; LCC6-WT – breast carcinoma, LCC6-MDR1 – PgP transfected line; HT-1080 – fibrosarcoma, HT-1080-DR4 – doxorubicin selected MDR (MRP/LRP phenotype).

[2]4.0 mM stock solutions that had been stored at 4°C for 7 days were used.

TX-001, TX-002 and paclitaxel were evaluated for tumor cell growth inhibition using a panel of human tumor cell lines including A121 ovarian, breast tumor cell lines LCC6 and the LCC6-MDR1 variant, which was transfected with the MDR1 gene and over-expresses P-glycoprotein. These compounds were also evaluated against an MRP/LRP expressing, multidrug resistance HT1080/DR4 human sarcoma cell line.

TX-001 and TX-002 are active agents against a number of human tumor cell types, with IC50 values ranging between 2.7-1798 nM (Table I). TX-001 appears to be more potent than TX-002 with a growth inhibition profile nearly identical to that of paclitaxel.

Resistance ratios (IC50 resistant cell line/IC50 parental line) in the LCC6-MDR1 variant line which overexpresses Pgp were similar for the three compounds tested. They ranged from 65 –92 fold for paclitaxel, 30 –100 fold for TX-001 and 47-60 fold for TX-002. Resistance ratios in the MRP/LRP expressing mutidrug resistant HT1080/DR4 cell line were also similar ranging from 1.1-1.4 fold for paclitaxel, 1.9-2.0 fold for TX-001 and 1.1-1.5 fold for TX-002.

Re-testing of TX-001 and TX-002 from stock solutions which had been stored at 4° C for 7 days, demonstrated a small increase of activity in LCC6 and LCC6-MDR1 cells. Testing with non-viscous stock solutions (0.4 mM) of TX-001 and TX-002 showed slightly less potency in all of the cell lines tested as compared to the initial results and repeat testing results of the 4.0 mM stock solutions.

Both TX-001 and TX-002 are active agents with TX-001 being the more potent.

The foregoing examples are illustrative of the present invention, and are not to be construed as limiting thereof. Rather, these embodiments are provided so that this disclosure will be thorough and complete, and will fully convey the scope of the invention to those skilled in the art. Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. The terminology used in the description of the invention herein is for the purpose of describing particular embodiments only and is not intended to be limiting of the invention.

All publications, patent applications, patents and other references cited herein are incorporated by reference in their entireties, as is the entire disclosure of each of the following references:

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